STRUCTURAL CHARACTERIZATION, BIOACTIVITY AND BIODEGRADATION OF CYANOBACTERIAL TOXINS

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Meiner Familie

GLOSSARY

Adda	*(2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid*
AG 828A	*Aeruginosin 828A*
Ahp	*3-amino-6-hydroxy-2-piperidone*
BMAA	*β-methyl-amino-L-alanine*
Choi	*2-carboxy-6-hydroxyoctahydroindole*
CP 1020	*Cyanopeptolin 1020*
DNA	*Deoxyribonucleic acid*
DOPA	*Dihydroxyphenylalanine*
GC-MS	*Gas chromatography-mass spectrometry*
GDM	*Gravity-driven membrane filtration*
GSH	*Glutathione*
GST	*Glutathione-s-transferase*
(H)PLA	*(Hydroxyl)phenyllactic acid*
HPLC	*High-performance liquid chromatography*
IC_{50}	*Half maximal inhibitory concentration*
i.p.	*Intraperitoneal (injection)*
LC_{50}	*Median lethal concentration*
LC-ESI-MS	*Liquid chromatography coupled to electrospray ionization mass spectrometry*
LC-MS	*Liquid chromatography-mass spectrometry*
LPS	*Lipopolysaccharid*
MC	*Microcystin*
Mdha	*Methyl-dehydroalanine*
NRPS	*Nonribosomal peptide synthase*
OATPs	*Organic anion transport proteins*
PKS	*Polyketide synthase*
ROS	*Reactive oxygen species*
SAR	*Structure-activity relationship*
VTG	*Vitellogenin*
WHO	*World health organisation*

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SUMMARY

Recurrent mass developments of cyanobacteria, so-called blooms, have become a matter of public concern as they are more and more frequently found in waters intended as recreational areas and drinking water. Many of the cyanobacterial species are known to produce toxins that have a variety of adverse effects, such as acute diarrhoea, skin irritation, liver damage, neurotoxicity, and even fatalities in humans a well as in domestic and wild animals. Yet, cyanobacterial impact on aquatic ecosystems can only be estimated, as knowledge about the effects of cyanobacterial bioactive compounds on aquatic organisms is rather limited. This thesis focused on three major topics: removal strategies for already known potentially toxic cyanobacterial peptides were evaluated; the chemical structure of a novel cyanobacterial toxin was elucidated; and, further studies included detailed characterizations of largely undescribed or novel toxins in order to evaluate their potential risk for humans and aquatic ecosystems in future.

Drinking water purification in terms of the elimination of cyanobacterial toxins is of major importance. Particularly in developing countries, simple and inexpensive purification systems are required. Gravity-driven membrane (GDM) ultrafiltration was shown to be an effective tool for the removal of pathogens and suspended solids by retention. We evaluated the biodegradation potential of the bacterial biofilm forming on the GDM ultrafiltration membrane for a cyanotoxin, microcystin. Successful elimination of microcystins in a GDM system required a maturation process of the biofilm, e.g. enrichment of bacteria capable of degrading microcystins. Phylogenetic analysis of the microbial communities forming the biofilm on the GDM ultrafiltration membrane gave first hints about potential new microcystin degraders.

Microcystins have long been considered as the most toxic and therefore most important cyanobacterial peptide class. Moreover, the investment of high amounts of resources into the production of microcystins suggests a very distinct and important function of these peptides for *Microcystis*, *Anabaena* and *Planktothrix*. Compounds being able to compensate the lack of microcystins were hypothesized. A screening of six *Planktothrix* strains with and without the ability to produce microcystins revealed the presence of a distinct peptide group, i.e. chlorinated and sulfated aeruginosins, in strains that lack microcystins. Structure elucidation of one representative of the chlorinated and sulfated aeruginosins, aeruginosin 828A, was conducted. Subsequent toxicological and enzymatic characterization revealed the discovery of a new cyanobacterial toxin. The toxicity of aeruginosin 828A towards the freshwater crustacean *Thamnocephalus platyurus* was in the range of microcystins (LC_{50} = 22.8 µM). Moreover, this peptide showed potent inhibition of serine proteases, e.g. IC_{50} = 112 nM for trypsin and IC_{50}= 21.8 nM for thrombin.

Chronic effects of the aeruginosin 828A in *Daphnia magna*, assessed during a 21 day life-history trait analysis and accompanied by the comparison of altered expression levels of selected genes, revealed pronounced effects on juvenile daphnids. Aeruginosin 828A caused severe moulting impairment and softening of the carapace, leading to reduced reproduction. However, adverse effects seemed to decrease once the animals had reached maturity. Expression levels of vitellogenin, a gene that is a well-known biomarker for estrogenic activity, were significantly decreased in juvenile *Daphnia* treated with aeruginosin 828A as compared to solvent controls. In mature animals, vitellogenin gene expression levels were unaltered. Transcriptome analysis of cyanopeptolin 1020 in zebrafish embryos gave first hints regarding possible modes of action and suggested cyanopeptolin 1020 to be associated with DNA-related activity and to have effects on the circadian rhythm.

Elucidation of the influence of cyanopeptolin 1020 and aeruginosin 828A on aquatic organisms and thus on the aquatic ecosystems may lead to a significantly altered understanding of the term “cyanobacterial toxin”. This may lead to a less anthropocentric perspective, but to a more ecotoxicological point of view in order to better understand the impact and function of cyanobacterial secondary metabolites.

ZUSAMMENFASSUNG

Das Massenauftreten von Cyanobakterien, sogenannte Blüten, sind von öffentlichem Interesse, seit diese Phänomene immer häufiger auch in Gewässern beobachtet werden, die als Erholungsraum oder als Trinkwasserreservoir genutzt werden. Viele Cyanobakterienarten produzieren Toxine, die für eine ganze Reihe von unangenehmen bis hin zu lebensgefährlichen Wirkungen bekannt sind. Akute Diarrhö, Hautirritationen, Leberschäden, Neurotoxizität und sogar tödliche Vergiftungen bei Menschen und Tieren können von diesen Substanzen verursacht werden. Über den Einfluss von bioaktiven Sekundärmetaboliten aus Cyanobakterien auf aquatische Organismen ist bis heute nur wenig bekannt; deshalb gibt es zu den Auswirkungen von Cyanobakterien und ihren Toxinen auf aquatische Ökosysteme bislang nur grobe Schätzungen. Daher hat die vorliegende Doktorarbeit drei Themenschwerpunkte bearbeitet: zum einen wurden Strategien zur Beseitigung von bekannten toxischen cyanobakteriellen Peptiden, Microcystin-LR, untersucht und evaluiert; im zweiten Teil der Arbeit wurde die chemische Struktur eines neuen cyanobakteriellen Toxins, Aeruginosin 828A, aufgeklärt; in weiterführenden Studien wurde dieses neue Toxin zusammen mit dem bislang nur begrenzt untersuchten cyanobakteriellen Toxin Cyanopeptolin 1020 weiter charakterisiert, um in Zukunft ihr potenzielles Risiko für Mensch und Umwelt abschätzen zu können.

Im Rahmen der Trinkwasseraufbereitung kommt der Beseitigung von cyanobakteriellen Toxinen grosse Bedeutung zu. Das sogenannte Gravity-Driven-Membrane (GDM) Ultrafiltrationssystem hält erfolgreich Pathogene und Schwebstoffe zurück und entfernt sie so aus dem Trinkwasser. In früheren Studien wurde gezeigt, dass sich auf der Oberfläche des GDM-Ultrafiltrationssystems ein bakterieller Biofilm ausbildet. Wir haben untersucht, in welchem Massstab dieser Biofilm in der Lage ist, Microcystine biologisch abzubauen. Die erfolgreiche Beseitigung von Microcystin aus dem GDM-System durch biologischen Abbau erfordert einen vorgängigen Reifeprozess des Biofilms, der die Anreicherung von Microcystin-abbauenden Bakterien beinhaltet. Phylogenetische Untersuchungen der bakteriellen Gemeinschaften, welche sich im Biofilm auf der GDM-Membran ausgebildet hatten, lieferten erste Hinweise über mögliche Microcystin-abbauende Bakterien.

Microcystine wurden aufgrund ihrer hohen Giftigkeit lange als Hauptproblem von Cyanobakterienblüten betrachtet und standen deshalb im Zentrum der Cyanobakterienforschung. Cyanobakterien investieren viele Ressourcen in die Produktion von Microcystinen. Dies weist darauf hin, dass diese Toxine eine wichtige Funktion für die Cyanobakterien erfüllen. Daher wird angenommen, dass Cyanobakterien auch Alternativstoffe produzieren, welche das Fehlen von Microcystinen kompensieren. Im Rahmen einer Vergleichsstudie von zwei Microcystin produzierenden mit vier nicht-produzierenden *Planktothrix* Stämmen wurden spezifische Chlor- und Sulfat-enthaltende Peptide - Aeruginosine - entdeckt, die nur in Stämmen produziert wurden, die keine Microcystine enthielten. Für einen Vertreter dieser

Peptidgruppe, Aeruginosin 828A, wurden Strukturaufklärungen und weitere toxikologische und enzymatische Untersuchungen durchgeführt. Aeruginosin 828A weist eine hohe Toxizität gegenüber *Thamnocephalus platyurus* auf (LC_{50} = 22.8 µM), die mit der von Microcystinen vergleichbar ist. Des Weiteren zeigte das neue Toxin starke Inhibition gegenüber den Serinproteasen Trypsin (IC_{50} = 112 nM) und Thrombin (IC_{50} = 21.8 nM).

Die Expressionsanalyse von ausgewählten Genen oder von ganzen Transkriptomen kann bei der Aufklärung von neuen Effekten, Signalwegen und Wirkweisen von Cyanotoxinen in *Crustaceae* und Fischen behilflich sein. Der Einfluss von Aeruginosin 828A auf *Daphnia magna* wurde im Rahmen einer 21 tägigen Lebenszyklusstudie untersucht und mit der Expression von ausgewählten Genen verglichen. Juvenile Daphnien zeigten dabei verstärkte Anfälligkeit gegenüber dem Toxin, wobei eine gewisse Aufweichung des Carapax und damit einhergehende Komplikationen bei der Häutung auftraten. Die damit einhergehende verminderte Reproduktionsfähigkeit ging im Verlaufe der Entwicklung jeweils wieder zurück. Im Rahmen der Genexpressionsanalyse mit *Daphnia magna* wurde Vitellogenin, ein Gen das als Biomarker für östrogene Aktivität bekannt ist, nur in juvenilen Daphnien runterreguliert. Transkriptom-Experimente mit Cyanopeptolin 1020 in Zebrafischen brachten erste Hinweise bezüglich möglicher Wirkungsweisen des Toxins. So wurden für Cyanopeptolin 1020 eine mögliche DNA-Aktivität und eine Wirkung auf den zirkadianen Rhythmus festgestellt.

Die neuen Erkenntnisse über diese Toxine bezüglich ihres Einflusses auf aquatische Organismen und damit einhergehende Konsequenzen für aquatische Ökosysteme könnten die Auffassung von „cyanobakteriellen Toxinen“ verändern. Eine stärkere Gewichtung der ökotoxikologischen Sichtweise anstelle einer anthropozentrischen Ausrichtung könnte in einem besseren Verständnis von Funktion und Wirkung von cyanobakteriellen Sekundärmetaboliten resultieren.

PREFACE

Within this dissertation, three chapters are comprehended that were summarized in four articles, whereof one (i) was published in *PLOSONE*, one (ii) was published in *Harmful Algae* (Kohler et al. 2014), one (iii) was published in *Aquatic Toxicology* (Faltermann et al. 2014) and one (iv) is currently being submitted to a toxicological journal. All four manuscripts are settled in the field of cyanobacteria and their toxic peptides by (i) investigating biodegradation potentials of cyanobacterial toxins and (ii-iv) by discovering new potential effects of selected cyanobacterial toxins.

The first chapter comprises the evaluation of a gravity-driven membrane (GDM) ultrafiltration system regarding the production of drinking water in the presence of a microcystin-containing cyanobacterial bloom.

Manuscript I: Esther Kohler; Jörg Villiger; Thomas Posch; Nicolas Derlon; Tanja Shabarova; Eberhard Morgenroth; Jakob Pernthaler; Judith F. Blom (2014) *Biodegradation of microcystins during gravity-driven membrane (GDM) ultrafiltration*.

The second chapter includes (ii) structure elucidation and enzymatic characterization of a new toxin, discovered in a screening of six cyanobacterial strains with and without the ability to produce microcystins.

Manuscript II: Esther Kohler; Verena Grundler; Daniel Häussinger; Rainer Kurmayer; Karl Gademann; Jakob Pernthaler; Judith F. Blom (2014) *The toxicity and enzyme activity of a chlorine and sulfate containing aeruginosin isolated from a non-microcystin-producing* Planktothrix *strain.*

The third chapter (iii-iv) comprises further investigations of cyanobacterial toxins regarding their effects on the aquatic model organisms zebrafish and *Daphnia magna*.

Manuscript III: Susanne Faltermann, Sara Zucchi, Esther Kohler, Judith F. Blom, Jakob Pernthaler, Karl Fent (2014) *Molecular effects of the cyanobacterial toxin cyanopeptolin (CP 1020) occurring in algal blooms: Global transcriptome analysis in zebrafish embryos.*

Manuscript IV: Esther Kohler, Judith F. Blom, Susanne Faltermann, Karl Fent, Jakob Pernthaler (in preparation). *Effects of aeruginosin 828A in comparison to microcystin-LR on life-history parameters and selected gene transcripts in* Daphnia magna.

In the following introduction, substantial background knowledge will be provided in order to bring all these studies in a broader context.

INTRODUCTION

1. CYANOBACTERIA – ECOLOGY AND ENVIRONMENTAL IMPACT

According to fossil records, cyanobacteria have been present on earth since no less than 3.5 billion years (Schopf, 1993). Moreover, they are suggested to be the first organisms on earth capable of performing oxygenic photosynthesis (Schwartz & Dayhoff, 1978), thus contributing considerably, if not primarily to the formation of the atmospheric oxygen (Mulkidjanian et al, 2006). Cyanobacteria are the only prokaryotic organisms on earth able to perform oxygenic photosynthesis and are therefore of evolutionary, environmental and geochemical importance (Gamalei & Scheremet'ev, 2013). Cyanobacteria have also led to plastids in photosynthetic eukaryotes since they have evolved from endosymbiotic cyanobacteria (Keeling, 2010). After all, cyanobacteria were originally classified as plants and termed blue-green algae due to their photosynthetic pigments chlorophyll a and the accessory pigments phycocyanin and phycoerythrin (Briand et al, 2003). These versatile organisms are distributed worldwide, from polar to equatorial regions, thereby being highly adapted to terrestrial and aquatic environments as unicellular organisms but also by forming symbioses within lichens and corals (Shevela et al, 2013). Cyanobacteria occur in manifold morphologies including spherical, ovoid and cylindrical unicellular species, as well as multi-cellular colonial and filamentous forms (Briand et al, 2003). Some species even form specialized cells: heterocysts allow for nitrogen fixation and akinetes are resting stages formed in adverse conditions (Adams & Duggan, 1999; Wolk et al, 2004).

Climate change as well as anthropogenic input of nutrients into aquatic environments has resulted in eutrophication of many lakes and rivers during the past century, and the increased nutrient availability favoured the accumulation of cyanobacteria at high densities, so called blooms (Figure 1), also in recreational areas or drinking water reservoirs (Paerl & Huisman, 2008; Posch et al, 2012).

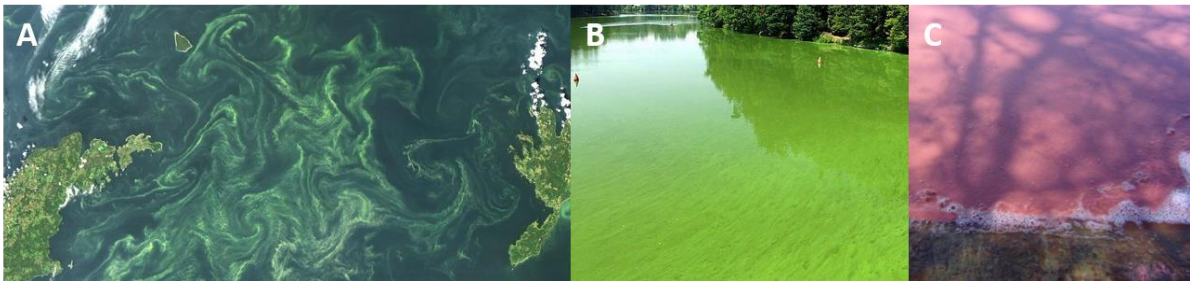

Figure 1. Cyanobacterial blooms. (A) Example of a bloom of *Nodularia spumigena* in the Baltic Sea, (B) *Microcystis aeruginosa* bloom in Sedlice Reservoir, Czech Republic, (C) *Planktothrix rubescens* bloom in Lake Hallwil, Switzerland [sources: M. Kahru, FytoPlankton.cz, J. F. Blom]

Cyanobacterial mass occurrences are not only undesirable due to unpleasant odours and tastes. Some of them might even produce noxious compounds (Falconer, 1999). The presence of cyanobacteria in waters has been associated with incidences of manifold intoxications and even fatalities of humans and animals (Briand et al, 2003; Carmichael, 2001; Francis, 1878).

2. CYANOBACTERIAL SECONDARY METABOLITES

Cyanobacteria are well known for the production of a wide variety of bioactive secondary metabolites that are mainly kept intracellularly. High amounts of these compounds might be liberated into the water during a collapse of a cyanobacterial bloom when cells undergo lysis (Codd, 2000; Huisman & Hulot, 2005; Zurawell et al, 2005). These substances span a vast diversity of effects on their environment. Bioactive metabolites that are typically found in freshwater cyanobacteria may be neurotoxic, hepatotoxic, cytotoxic or dermatotoxic (irritant toxin) (Pearson et al, 2010). Classification of those compounds is typically done in accordance to their chemical structure. Therefore, they may be assigned to alkaloids, amino acid derivatives (such as BMAA), lipopolysaccharids (LPS) and peptides (Pearson et al, 2010; Vasas et al, 2010). The majority of those secondary metabolites were found to be produced by polyketide synthase (PKS), non-ribosomal peptide synthase (NRPS) pathways or through modifications of ribosomally produced peptides (Dittmann et al, 2013; Méjean & Ploux, 2013; Rounge et al, 2009). The production of cyanobacterial toxins is thus genetically determined and may lead to parallel occurrences of different derivatives of the same substance class in a single cyanobacterial strain. This phenomenon is particularly well described for cyanobacterial peptides. Moreover, strain specific peptide patterns, referred to as chemotypes, are well known to remain constant regardless of changing temperature, light or macronutrient concentrations (Rohrlack et al, 2008; Welker et al, 2004b).

Alkaloids: Cyanobacterial alkaloids such as (homo-)anatoxin a, anatoxin a(*S*), saxitoxin, cylindrospermopsin, and other toxins such as indole alkaloids may typically provoke different neurotoxic, hepatotoxic or cytotoxic effects. The neurotoxic anatoxins were detected in *Anabaena flos-aquae*, *A. circinalis*, *Aphanizomenon* sp., *Cylindrospermum* sp., *Planktothrix* sp. and *Microcystis aeruginosa* (Edwards et al, 1992; Park et al, 1993; Sivonen et al, 1989). They are well known for blocking neuromuscular transmission due to irreversibly binding to the nicotinic acetylcholine receptor or the acetylcholinesterase (Wiegand & Pflugmacher, 2005). The paralytic shellfish poison saxitoxin is an ion channel blocker and thus neurotoxic. It is primarily produced by marine dinoflagellates but also by cyanobacterial species like *Aphanizomenon* sp., *Anabaena* sp., *Lyngbya* sp., and *Cylindrospermum raciborskii* (Humpage et al, 1994); Cylindrospermopsin is inhibiting protein synthesis and may lead to DNA strand breaks and thus cause hepatotoxic, neurotoxic and cytotoxic effects (Vasas et al, 2010). It was found in *Cylindrospermum raciborskii*, *Umezakia natans*, *Aphnizomenon ovalisporum*, *Rhaphidiopsis curvata*

and *Anabaena bergii* (Banker et al, 1997; Harada et al, 1994; Li et al, 2001; Ohtani et al, 1992; Schembri et al, 2001). Indole alkaloids are manifold and were associated with antiviral, antimicrobial and anti-fungal activity as well as with cytotoxicity (Kim et al, 2012).

Amino acid derivatives: β-methyl-amino-L-alanine (BMAA) was proposed as a cause of amyotrophic lateral sclerosis, however, its mode of action is not yet understood (Pearson et al, 2010; Vasas et al, 2010).

LPS: Cyanobacteria belong to the gram-negative bacteria and thus possess endotoxic lipopolysaccharids (LPS) as part of their outer cell layer. LPS are fatty acid components that affect the immune system and the detoxification systems of different organisms, thereby decreasing the ability of organisms to metabolize xenobiotics, including cyanobacterial toxins. LPS belong to the irritant toxins, however, their contribution to cyanobacterial toxicity is not fully understood (Wiegand & Pflugmacher, 2005).

Peptides: Collapses of cyanobacterial blooms are followed by a release of considerable amounts of cyanobacterial peptides. Therefore, much attention is paid to the identification of harmful or bioactive compounds with peptide characteristics. Many cyclic and few linear oligopeptides induce various bioactive effects such as enzyme inhibition or acute toxicity to pro-and eukaryotes (Chlipala et al, 2011). A wide variety of different techniques has been used for structure elucidation and toxicity assessment of several hundreds of these compounds. Most of these peptides could be assigned to seven distinct peptide classes: microcystins, cyanopeptolins, anabaenopeptins, aeruginosins, cyclamids, microginins and microviridines (Welker & von Döhren, 2006).

3. CYANOBACTERIAL PEPTIDES

Cyanobacterial oligopeptides are well known for their diverse bioactivities. Research has been promoted by two interest groups: on the one hand, the pharmaceutical industry has discovered cyanobacteria as a promising source for potential prospective pharmaceuticals; on the other hand, human health authorities are concerned about cyanotoxins and their potential to affect human health (Agha & Quesada, 2014; WHO, 2011).

3.1. MICROCYSTINS – THE BEST STUDIED CYANOTOXINS

Microcystins (MCs) that are mainly produced by *Anabaena* sp., *Microcystis* sp., *Planktothrix* sp. (formerly classified as *Oscillatoria* sp.), *Aphanizomenon* sp., and from terrestrial *Hapalosiphon* sp. have been in the focus as they are highly toxic even in very low concentrations not only to their herbivorous predators but also to other organisms, including humans (Campos & Vasconcelos, 2010; Codd et al, 2005; Dawson, 1998). MCs are cyclic heptapeptides consisting of the characteristic unusual amino acid (2S,3S,8S,9S)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid (*Adda*) at position 5

and six further amino acids including glutamate at position 6, aspartate derivative at position 3, alanine in position 1, methyl-dehydroalanine (*Mdha*) in position 7, whereas, positions 2 and 4 are less conserved (Figure 2). Due to this high variability, the nomenclature of the MCs names them according to the amino acids in position 2 and 4 by setting the one letter code for amino acids, e.g. LR. Thus, the general structure of MCs is cyclo-(D-Ala^1-X^2-D-MeAsp^3-Z^4-Adda^5-D-Glu^6-Mdha^7), with X and Z denoting the highly variable L-amino acids (Carmichael et al, 1988).

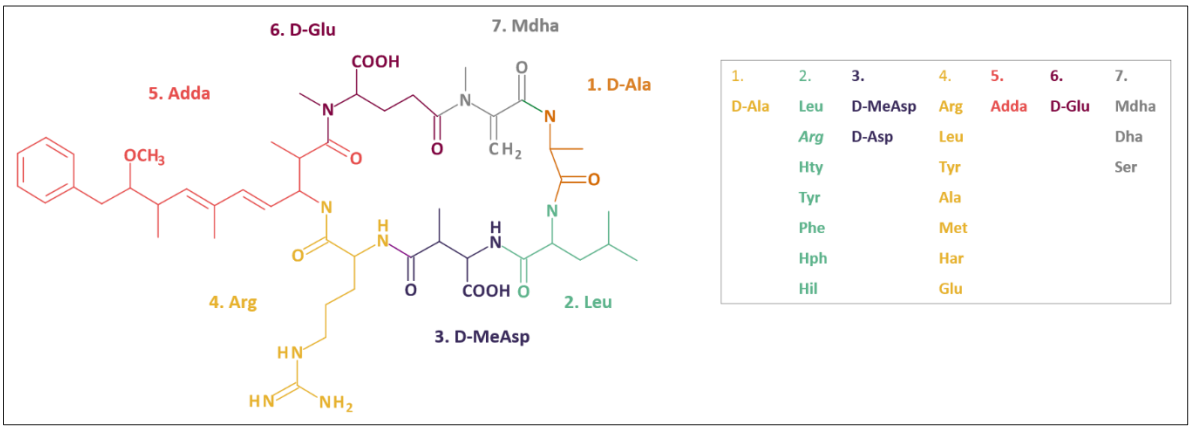

Figure 2. Microcystin. Structure of microcystin-LR and schematic general structure of microcystin type peptides. Note that the numbering does not correspond to the suite of biosynthetical steps that starts with 5 and ends with 4. Modified after (Welker & von Döhren, 2006).

Biosynthesis of MCs requires an intact *mcy* gene cluster of at least two operons including *mcy*-ABC (NRPS) and *mcy*DE (PKS) genes and varies among cyanobacterial genera (Tillett et al, 2000) (Figure 3). Around 90 variants of MCs have been identified yet and MC-LR was shown to be the most common and very toxic derivative (Neilan et al, 2013; Welker & von Döhren, 2006).

Figure 3. Microcystin biosynthesis. MCs are produced by a multi-enzyme complex comprised of non-ribosomal peptide synthase (NRPS) and polyketide synthase (PKS) units, and other enzymes such as tailoring enzymes. All are encoded by the *mcy*A-J cluster. Differences in the construction of the gene-clusters seem to be strain specific. Modified after (Kalaitzis et al, 2009).

These toxic oligopeptides are strong inhibitors of protein phosphatases of types 1 or 2A (Dawson, 1998). However, toxicity of the MC derivative does not necessarily correlate with inhibition potential for protein phosphatase (Blom & Jüttner, 2005). Inhibition of these phosphatases was made responsible for the acute toxicity of MCs, as it may lead to hyperphosphorylation of proteins in cells and to deformation of the cytoskeleton. When MCs are ingested, they are transported via bile acid transport to the liver and by organic anion transport proteins (OATPs) into the hepatocytes (Fischer et al, 2005). This may eventually lead to intra-hepatic haemorrhage or hepatic insufficiency and even

haemorrhagic shock (Briand et al, 2003; Fischer et al, 2005; Neilan et al, 2013). MCs are also responsible for the increased production of reactive oxygen species (ROS), thus inducing oxidative stress in cells and by this triggering apoptosis (Campos & Vasconcelos, 2010). Moreover, MCs were shown to cause alterations of liver tissue, including endoplasmic reticulum stress and chronic inflammation and even act as tumour promoters by DNA-alteration, when administered in lower concentrations (Christen et al, 2013; Humpage & Falconer, 1999; Rao & Bhattacharya, 1996). However, the toxicity of different MCs is quite variable, ranging from highly toxic like MC-LR with an LD_{50} = 50 µg kg^{-1} bodyweight in mice (*i.p.*) to low toxic like MC-RR with an LD_{50} = 600 µg kg^{-1} bodyweight in mice (*i.p.*) (Rinehart et al, 1994). Detoxification of MCs was suggested to occur by conjugation of MCs to glutathione (GSH) via glutathione-s-transferase (GST) (Buratti et al, 2011). Moreover, the involvement of cytochrome p450 was hypothesized (Zegura et al, 2011). However, details of the pathways and enzymes involved in metabolism and excretion of MCs are still lacking (Campos & Vasconcelos, 2010; Pflugmacher et al, 1998).

Only few human fatalities due to MC poisoning have been reported (Jochimsen et al, 1998; Ueno et al, 1996). However, many effects on human health are known: hepatotoxicity, kidney disorders, gastrointestinal impairment, allergic and irritation reactions as well as eye, ear and skin irritation, and nausea, vomiting and diarrhoea (Rastogi et al, 2014). The majority of MC poisonings of terrestrial vertebrates, including humans, is associated with MC uptake by dermal contact with MC-containing water during swimming or its accidental consumption, or even more by consuming untreated contaminated water or food (Papadimitriou et al, 2012; Rastogi et al, 2014). Moreover, an uptake route via inhalation of aerosolized MCs has been proposed (Cheng et al, 2007).

3.2. Microcystins In Drinking Water

The severe threat of MCs to humans led the World Health Organization (WHO) to propose a guideline value of 1.0 µg L^{-1} for the concentration of MC-LR, the most common and very toxic MC variant, in drinking water, as suggested by Falconer et al. (1994), (WHO, 2011). Therefore, measures for the removal of MCs from waters were taken in many countries. However, removing MCs from drinking water is a difficult task, since MCs are very persistent in natural waters, withstanding sunlight, a range of pH and temperatures, and moreover, are insusceptible to hydrolization and oxidation (Harada et al, 1996; Tsuji et al, 1994; Tsuji et al, 1997; Tsuji et al, 1995). Effective removal of MCs was achieved by application of powdered activated carbon (Campinas & Rosa, 2010), sediment sorption (Grutzmacher et al, 2010), or ozonisation (Hoeger et al, 2002). To date, many countries have taken measures to successfully reduce or eliminate cyanotoxins in contaminated waters destined for drinking (Chorus, 2005).

In developing countries, expert knowhow regarding drinking water purification may be limited. Moreover, financial and energy resources are often insufficient for the establishment and maintenance

of conventional centralized drinking water production that are commonly found in developed countries. Alternatively, several supposedly suitable water treatment strategies have been suggested (Fewtrell et al, 2005). Membrane filtration excels as a sustainable method for simple, inexpensive drinking water production, leading to readily available purified water without the need of constant maintenance or chemicals (Gijsbertsen-Abrahamse et al, 2006; Peter-Varbanets et al, 2009). Gravity driven membrane (GDM) ultrafiltration was proven an effective method to retain pathogens, disease vectors and suspended solids, and, as a great bonus, is operated electricity-free (Derlon et al, 2013; Peter-Varbanets et al, 2009).

Microbial biodegradation of MCs is considered a good approach to remove the toxicity of MCs as several bacteria are known for their potential to efficiently biodegrade these toxins with their specialized enzymes (Dziga et al, 2013; Rastogi et al, 2014). So far, only a few studies have tried to link the composition of bacterial communities in biofilms of biological drinking water treatment with the ability of these systems to degrade microcystins (Shimizu et al, 2013).

3.3. AERUGINOSINS

The aeruginosin peptide class was first described by Murakami et al. (1995) and is characterized by a linear derivative with the common motif of a (hydroxyl)phenyllactic acid (*Hpla/Pla*) or glyceric acid at the N-terminus in position 1, followed by a variable amino acid at position 2, the 2-carboxy-6-hydroxyoctahydroindole (*Choi)* moiety at position 3, and an arginine derivative at the C-terminus in position 4 (Figure 4). Chlorination and sulfation are regularly observed at the *(H)Pla* and at the *Choi* moiety. Chlorination may additionally be found at the amino acid in position 2 (Hanessian et al, 2009; Welker & von Döhren, 2006). Likewise, glycosylation at the Choi moiety with xylose is common in aeruginosins.

Aeruginosin biosynthesis is conducted via NRPS/PKS pathways including tailoring enzymes such as halogenases and sulfotransferases. More than 500 different derivatives may be conceivable by the high structural variability of the corresponding *aer* gene-cluster. Yet, only around 30 predominant aeruginosin derivatives have been described in *Planktothrix* and *Microcystis (Ersmark et al, 2008; Nagarajan et al, 2013)* . In addition, structural similarities are found in dysinosins isolated from a dysideid sponge, as well as suomilides found in *Nodularia,* and in banyasides isolated from *Nostoc* (Carroll et al, 2004; Carroll et al, 2002; Fujii et al, 1997; Pluotno & Carmeli, 2005).

Aeruginosins are known as potential trypsin and thrombin inhibitors, and thus were referred to as promising drug precursors. Likewise, aeruginosin derivatives were used as a model for the creation of synthetic thrombin inhibitors leading to treatment of deep vein thrombosis, myocardial infarction and stroke (Ersmark et al, 2008; Radau et al, 2003). Moreover, anti-inflammatory effects in human

endothelial cells were found in distinct aeruginosin derivatives (Kapuscik et al, 2013). In mammals, no toxicity has been associated with aeruginosins yet.

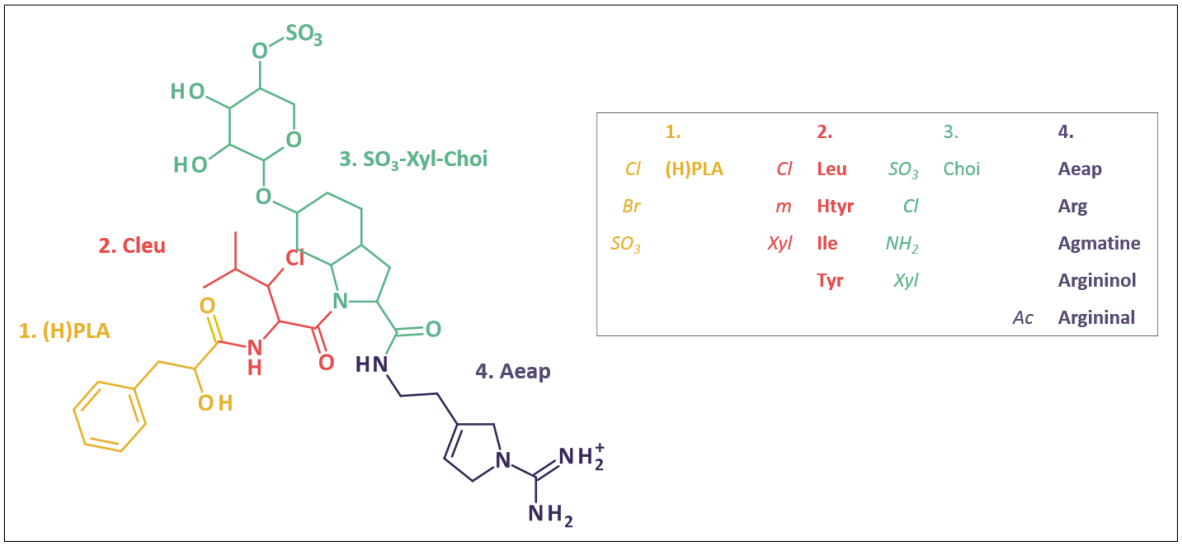

Figure 4. Aeruginosin 828A. The basic linear structure containing four moieties: (hydroxyl)phenyllactic acid (Hpla/Pla) or glyceric acid at the N-terminus, followed by a variable amino acid, the Choi moiety, and an arginine derivative. With possible modifications [Cl, chlorination; Br, bromination; SO_3, sulfation; m, O-methylation; Xyl, glycosylation; NH_2, amination; Ac, acylation]. Modified after (Welker & von Döhren, 2006).

3.4. CYANOPEPTOLINS

Cyanopeptolins are found in different cyanobacterial genera like *Microcystis*, *Planktothrix* and *Anabaena*, representing another non-ribosomally produced cyanobacterial peptide class toxic towards aquatic crustaceans. The cyclization of this depsipeptide is achieved by an ester bond of the β-hydroxy group of threonine with the carboxy group of the terminal amino acid. Cyanopeptolins contain an unusual 3-amino-6-hydroxy-2-piperidone unit (*Ahp*) and a lactone bond, thus belonging to the class of branched peptidolactones. Sulfation, chlorination and O-methylation are commonly observed in cyanopeptolins (Chlipala et al, 2011), resulting in a high variability of this structurally rather diverse peptide class with so far over 120 described derivatives from freshwater and terrestrial cyanobacteria (Figure 5). A variety of synonyms are used for cyanopeptolins, due to the simultaneous publishing of several new structures of cyanopeptolin derivatives: aeruginopeptin, anabaenopeptilide, microcystilide, micropeptin, nostocyclin, nostopeptin, oscillapeptilide, oscillapeptin, planktopeptin (Welker & von Döhren, 2006).

Nonribosomal peptide synthesis of cyanopeptolins was associated with distinct gene-clusters in *Anabaena* sp., *Microcystis* sp. and *Planktothrix* sp. (*apd*, *mcn* and *oci*) in addition to several mainly strain specific tailoring domains like methyltransferases, sulfotransferases, halogenases, formyl transferases and glyceric acid transferases (Rounge et al, 2008).

Very potent inhibitors of serine proteases like trypsin and chymotrypsin are found among cyanopeptolins. Since many pathological mechanisms involve proteases, e.g. viral proteases, these inhibitors of digestive proteases might be useful for the discovery and development of pharmaceuticals (Chlipala et al, 2011).

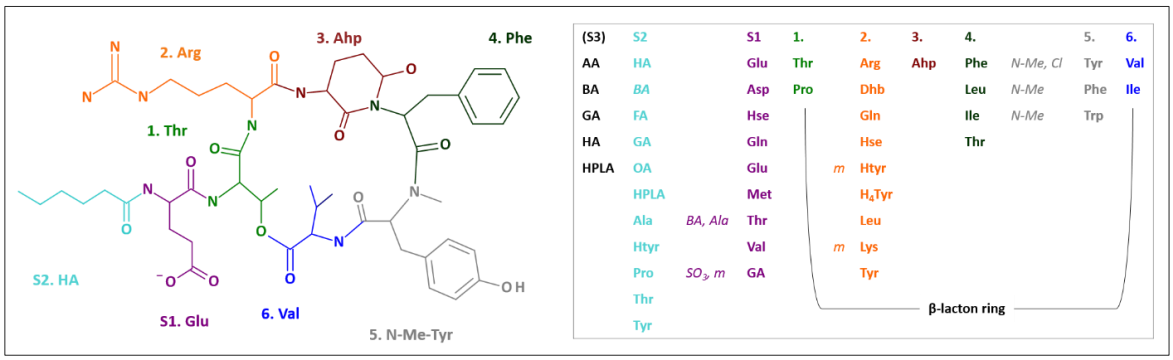

Figure 5. Cyanopeptolin. Structure of cyanopeptolin 1020 and schematic general structure of cyanopeptolin type peptides. GA, glyceric acid; FA, formic acid; AA, acetic acid; BA, butanoic acid; HA, hexanoic acid; OA, octanoic acid; HPLA, hydroxyphenyllactic acid; Cl, chlorination; N-Me, a-aminomethylation; m, O-methylation; SO_3, sulfation. The numbering of the side chain is in reverse order. Modified after (Welker & von Döhren, 2006).

4. The Ecological Role Of Cyanobacterial Peptides

Cyanobacterial peptide production has been preserved over long evolutionary periods despite its metabolic costs. Therefore, it seems likely that these compounds have important biological functions rather than just being waste products (Demain & Fang, 2000). Next to regulatory roles, secondary metabolites have been suggested to provide competitive advantages to their producers (Holland & Kinnear, 2013). Considering the long presence of cyanobacteria on earth, their evolutionary adaptations to changing environments have been manifold. The production of distinct secondary metabolites might have provided these organisms with competitive advantages that may be obsolete in present-day environments. Thus, these persistently produced secondary metabolites may not be relevant in modern aquatic environments anymore or else, have lost their original function but are beneficial in the new situation. It is also conceivable that cyanobacterial oligopeptides fulfil more than one function, comparable to the antibiotics produced by microbial species (Demain & Fang, 2000). Considering the broad spectrum of harmful effects of cyanobacterial oligopeptides on many aquatic and terrestrial organisms, it seems inviting to conclude that cyanobacterial peptide production was mostly aiming for protection against predators and resource competitors. However, the apparent toxic properties of cyanobacterial peptides may be completely unrelated to their original function. For MCs, several biological functions were suggested, including involvement in photosynthesis, metal chelator activity, a role as infochemicals and regulation of gene expression (Schatz et al, 2007). Even allelopathic functions

were attributed to MCs, giving their owners distinct advantages in terms of resource competition (Singh et al, 2005).

4.1. Microcystins As Grazer Defence

Potential evolutionary competitive advantages imply that toxin production may have originated from the need for a cellular defence mechanism, in response to grazing pressure or resource competition (Holland & Kinnear, 2013). The effects of cyanobacteria on naturally co-occurring aquatic organisms and vice versa have been the subject of many studies (Freitas et al, 2014). A cyanobacterial diet was shown to have a negative impact on zooplankton in general and particularly on *Daphnia* spp., a key-stone species in aquatic ecosystems (Sarnelle, 2005). *Daphnia* spp. were found to suffer from impediments during ingestion of cyanobacteria, especially due to cyanobacterial aggregate formation mechanically affecting the filter-apparatus, but non-mechanical ingestion inhibition and acute toxicity was also observed. Likewise, cyanobacteria were shown to adversely affect several fish species and were attributed with impairments in gills, spleen, heart, kidney, skin, digestive tracts and liver (Chorus, 1999). Many of the described negative effects of cyanobacteria on fish and crustacean were eventually attributed to the presence of cyanobacterial oligopeptides. While detailed investigations regarding the properties of these compounds revealed diverse bioactivities, MCs were generally agreed on constituting the major toxic agents within the plethora of cyanobacterial secondary metabolites (Christoffersen, 1996). Experiments with pure MCs showed severe acute toxicity in zooplankton such as *Daphnia* sp, *Eudiaptomus gracilis*, or *Thamnocephalus platyurus*, but also feeding inhibition, reduction in growth and in reproductive success were observed (Kurmayer & Jüttner, 1999; Rohrlack et al, 2005). Likewise, MCs severely affect growth, survival and physiological functions in several fish and amphibian species (Oberemm et al, 1999). Therefore, MCs were suggested as major cyanobacterial defences against their herbivorous predators (DeMott et al, 1991).

4.2. Microcystin-Deficient Strains

Planktothrix blooms in many cases consist of a vast variety of different cyanobacterial chemotypes, including a large proportion of individuals that do not produce MCs (Ostermaier & Kurmayer, 2009). A lack of MC production may occur due to diverse mutations (insertions or deletions) in the MC synthase (*mcy*) gene cluster (Figure 6).

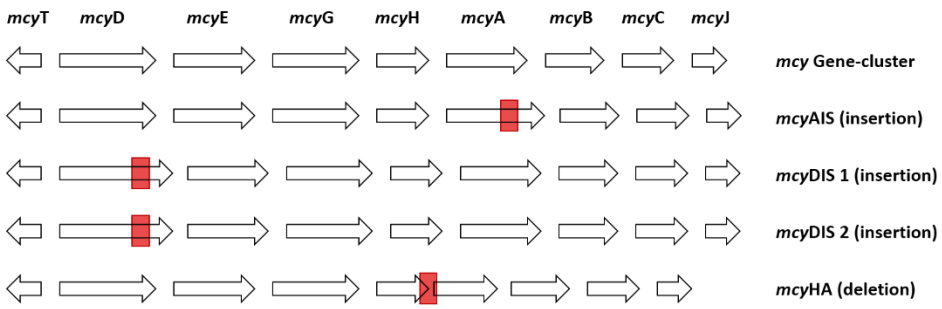

Figure 6. MC synthase (*mcy*) gene cluster of *Planktothrix*. Inactivation of the *mcy* gene cluster is caused by insertion (*mcy*AIS, *mcy*DIS1, *mcy*DIS2) or deletion of parts of *mcy*A and *mcy*H (*mcy*HA) (Ostermaier & Kurmayer, 2009).

The inactivation of the *mcy* gene cluster has occurred in relatively recent times but cannot be assigned to distinct phylogenetic lineages as reflected by several variable genetic markers (Christiansen et al, 2006). The loss of an active *mcy* seems to be of no disadvantage for the success of the strains lacking MCs. A linear relation of MC-deficient strains to the total population density was found for the bloom forming *Planktothrix* populations in European lakes (Ostermaier & Kurmayer, 2009). The percentage of MC-producers and non-producers in a perennial bloom was found to be rather variable and the function of factors promoting toxic over nontoxic strains and vice versa remain unclear (Orr & Jones, 1998; Schatz et al, 2005). A comparison of cyanobacterial strains with and without the ability to produce MCs was suggested to discover mechanisms compensating for the lack of MC. Additionally, this knowledge would probably be crucial for the elucidation of MC function.

MCs have mainly been made responsible for cyanobacterial toxicity towards zooplankton and fish until MC-deficient strains were found to cause toxicity in these organisms as well (Kaebernick et al, 2001; Rogers et al, 2011). Moreover, studies including MC-deficient strains revealed acute toxic effects and also reduced reproduction success during chronic exposure in *Daphnia* sp. (Hulot et al, 2012). In addition, endocrine disrupting agents that were not related to MCs were found during global gene expression profiling in zebrafish (Rogers et al, 2011).

Davies and co-workers (2011) compared the impact of *Microcystis* with and without the ability to produce MCs on zooplankton and found both being equally grazed. Therefore, toxic peptides other than MCs were hypothesised to be present in cyanobacteria, causing similar grazing inhibitions as MCs. Clearly, there are peptides other than MCs potentially exhibiting adverse effects on aquatic organism. Microviridin J was shown to prevent hardening of carapace in *Daphnia* sp., thus leading to high mortality. Serine protease inhibitory properties of the compound were suggested to be responsible for the observed effects, leading to incomplete protein digestion and consequently a lack of amino acids, that may be essential components of the integument of daphnid. Alternatively, direct interference of microviridin J with moulting fluid was suggested to lead to incomplete moulting (Kaebernick et al, 2001; Rohrlack et al, 2003). Also cyanopeptolins have been attributed with high acute toxicity in zooplankton and serine protease inhibition (Blom et al, 2006; Blom et al, 2003; Gademann et al, 2010). However,

microviridin J and cyanopeptolins are present in cyanobacteria independent of the presence of MCs (Kurmayer et al, 2014). Thus, they may not compensate the lack of MCs but rather supplement it.

5. AIMS OF THE THESIS

Production of safe drinking water in developing countries is a difficult task. Limitation in resources and knowledge require simple and inexpensive solutions like the GDM ultrafiltration system. In the first chapter of this thesis, we aimed for the examination of possible degradation of MCs by the microbial biofilm forming on a GDM ultrafiltration membrane. We simulated an artificial cyanobacterial bloom and its collapse, and we determined the ability of the microbial biofilm to remove MCs during drinking water production. Analysis of the bacterial assemblages of the biofilms in the GDM ultrafiltration system by next generation sequence analyses was conducted in order to obtain information about the microorganisms that might potentially be involved in removal of MCs.

It is generally believed that oligopeptide production is essential for cyanobacteria and that maintaining a high diversity of oligopeptides from one or several chemical classes is of advantage to overcome competition by other phytoplankton organisms or grazing pressure by herbivores. In a perennial cyanobacterial bloom, individuals with and without the ability to produce MCs are commonly found. MC-deficient strains were omnipresent, without showing obvious disadvantages due to the lack of MCs. In order to explain these findings it was suggested that an alternative peptide or peptide class might functionally compensate for the lack of MCs.

In the second chapter, a *Planktothrix* culture collection comprising two MC-producing and four MC-deficient strains was assessed by bioassay-guided fractionation with the sensitive crustacean *Thamnocephalus platyurus.* Toxic fractions were subject to further analyses and revealed the presence of novel cyanobacterial toxins. One representative of the novel toxins was isolated and purified in high amounts in order to conduct structure elucidation as well as enzymatic and toxicological characterisations.

Knowledge about the potential risk of cyanobacterial serine protease inhibitors for aquatic ecosystems is rather limited. Global transcriptome analysis, proteomics and life-history-trait-analysis provide effective strategies and tools for ecotoxicological studies to elucidate symptoms and mechanisms of actions upon exposure.

In the third chapter, on the one hand, transcriptome analysis of zebrafish, exposed to cyanopeptolin 1020 was carried out, in order to get first hints regarding possible molecular effects and even mode of action. On the other hand, comparison of effects of CP 1020, MC-LR and the newly discovered toxin on *Daphnia magna*, including analysis of the expression of selected genes and life-history trait

analysis aimed at the elucidation of ecotoxicologically relevant effects. Moreover, these studies were performed to give valuable input in the ongoing discussion about the ecological relevance and functions of cyanobacterial peptides.

CHAPTER 1 - DRINKING WATER

MANUSCRIPT I

Esther Kohler; Jörg Villiger; Thomas Posch; Nicolas Derlon; Tanja Shabarova; Eberhard Morgenroth; Jakob Pernthaler; Judith F. Blom (2014) *Biodegradation of microcystins during gravity-driven membrane (GDM) ultrafiltration*. Plos One 9: e111794

MANUSCRIPT I

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Biodegradation of Microcystins during Gravity-Driven Membrane (GDM) Ultrafiltration

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Abstract

Gravity-driven membrane (GDM) ultrafiltration systems require little maintenance: they operate without electricity at ultra-low pressure in dead-end mode and without control of the biofilm formation. These systems are already in use for water purification in some regions of the world where adequate treatment and distribution of drinking water is not readily available. However, many water bodies worldwide exhibit harmful blooms of cyanobacteria that severely lower the water quality due to the production of toxic microcystins (MCs). We studied the performance of a GDM system during an artificial *Microcystis aeruginosa* bloom in lake water and its simulated collapse (i.e., the massive release of microcystins) over a period of 21 days. Presence of live or destroyed cyanobacterial cells in the feed water decreased the permeate flux in the *Microcystis* treatments considerably. At the same time, the microbial biofilms on the filter membranes could successfully reduce the amount of microcystins in the filtrate below the critical threshold concentration of 1 μg L^{-1} MC for human consumption in three out of four replicates after 15 days. We found pronounced differences in the composition of bacterial communities of the biofilms on the filter membranes. Bacterial genera that could be related to microcystin degradation substantially enriched in the biofilms amended with microcystin-containing cyanobacteria. In addition to bacteria previously characterized as microcystin degraders, members of other bacterial clades potentially involved in MC degradation could be identified.

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Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper and its Supporting Information files.

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Introduction

During the last century, the anthropogenic input of nutrients into freshwaters has resulted in a distinct increase of cyanobacterial biomass in many water bodies worldwide [1]. Climate change and global warming may even increase the frequency and intensity of cyanobacterial blooms in the future [2]. Some cyanobacteria represent a major challenge for drinking water usage due to their production of microcystins (MCs), toxic secondary metabolites that affect a wide range of animals and humans [3]. The major route of human exposure to MCs is via oral ingestion, mainly due to the consumption of drinking water [4]. Even a subchronic dose of MCs in drinking water may elevate the rate of liver cancer, as was shown in China, where prevalent incidences of liver cancer correlated with MC-contaminations in drinking water [5]. Consequently, the WHO has developed a guideline for MCs in drinking water stating that an average exposure generally should be below the level of 1 μg L^{-1} [4]. *Microcystis aeruginosa* is known to form massive blooms in many lakes worldwide, and it produces MCs in high amounts. The concentrations of the intracellular MCs range between 0.3 to 15 μg L^{-1} [6] and up to 400 μg L^{-1} [7] in cyanobacterial blooms, however, occasionally high concentrations of up to 1400 μg L^{-1} were found [8]. Elimination of the MCs from drinking water is therefore highly desirable.

The largely cell-bound MCs are eliminated by removing the intact cyanobacterial cells by conventional water treatment procedures such as coagulation or flocculation [4]. However, cell damage (e.g. during the collapse of a bloom) will release toxins into water, and the above mentioned procedures will not sufficiently remove MCs from drinking water. Strategies such as powdered activated carbon [9], sediment sorption [10], or ozonisation [11] have been suggested to effectively eliminate dissolved MCs. However, these treatments are costly in terms of development and management (energy, need of chemicals) and thus not suitable for the application in developing and transient countries.

Gravity driven membrane (GDM) ultrafiltration is considered for drinking water production as a relevant alternative to common appliances [12]. GDM uses a simple set-up [13], which is inexpensive, electricity-free, easy to use, and it is already known to provide an effective barrier against pathogens, disease vectors and suspended solids [14]. Microbial activity as well as the total

organic carbon content in the feed water have been shown to affect the performance of the GDM ultrafiltration without control of the biofilm formation (no backwashing or chemical cleaning) [13]. However, nothing is currently known about the possible degradation processes of intact cyanobacterial cells or of toxins such as MCs in these point-of-use membrane systems. In recent years, biodegradation by heterotrophic bacteria has been recognized as an alternative way to eliminate MCs [15]. A few bacterial isolates capable of MC degradation have been already characterized [16]. The best studied MC degrading bacteria are belonging to the *Alphaproteobacteria* such as *Sphingomonas* sp. [17], *Sphingopyxis* sp. [18], or *Novosphingobium* sp. [19]. However, only a few studies have tried to link the composition of bacterial communities in plankton [20] or in biofilms of biological drinking water treatment facilities [21] with the ability of these systems to degrade microcystins.

The objective of our study was to examine possible degradation of MCs by the microbial biofilm of a GDM ultrafiltration system. We simulated cyanobacterial blooms and their collapse (and thus the release of the cell-bound MCs into the surrounding water) and determined the ability of the microbial biofilm to remove MCs during drinking water production. We also analysed the composition of microbial assemblages of the biofilms of the GDM ultrafiltration systems by next generation sequence analyses in order to obtain information about the microorganisms that might potentially be involved in this process.

Materials and Methods

Cyanobacterial cultures and quantification of microcystins

Axenic cultures of Microcystis aeruginosa PCC 7806 were kept at 20°C in Cyano-medium in several Erlenmeyer flasks under constant light at 5 µmol quanta m^{-2} s^{-1} from fluorescent tubes. Fresh Microcystis aeruginosa PCC 7806 cultures were taken every three to four days from the cultivation for the ongoing experiment. The cell number of the cyanobacterial culture was determined by flow cytometry (described below), and the MC concentration was quantified by high-performance liquid-chromatography (HPLC) as followed: A volume of 5 mL of the culture was frozen at −23°C for three hours. After thawing, 7.5 mL of 100% methanol (MeOH) were added to achieve a 60% aqueous methanolic solution. The extract was centrifuged for 15 min at 25′700 g. HPLC analysis was performed on a Shimadzu 10 AVP system with photodiode array detector (PDA) and a Hydrosphere C18 column (YMC, 4.6×250 mm, Stagroma, Switzerland), using solvent A: UV-treated H_2O containing 0.05% trifluoroacetic acid (TFA, Merck) and solvent B: acetonitrile and 0.05% TFA. A gradient was achieved by applying linear increases in two steps (solvent B from 35% to 70% in 30 min, 70% to 100% in 2 min). For the quantification procedure, calibration curves for MC-LR and [D-Asp^3] MC-LR, the two MCs of M. aeruginosa PCC 7806 had to be established: The two MCs were isolated in high purity (>99%, HPLC) from Microcystis aeruginosa PCC 7806, and their specific molar absorption coefficient was used to prepare accurate standard solutions between 1 and 10 µg mL^{-1}. The calibration curves were based on the peak area recorded at a wavelength of 239 nm. The microcystin quantification was done in duplicate and is referred to as the sum of the concentrations of MC-LR and [D-Asp^3] MC-LR.

Experimental setup of the Gravity-Driven-Membrane (GDM) system

Water from a depth of 5 m of Lake Zurich was continuously pumped by a fountain pump (Nautilus 450, Oase GmbH, Hörstel, Germany) to a storage tank (6 l volume, kept in the dark at room temperature). This storage tank was connected by silicon tubes (Saint-Gobin) to six parallel membrane modules consisting of filter holders of 48 mm inner diameter (Whatman, Maidstone, Kent, UK) and polyethersulfone ultrafiltration membranes with a 150 kDa nominal cut-off (PBHK, Biomax Millipore, Billerica, MA, USA). A hydrostatic pressure of 0.65 mbar was received by keeping the storage tank 0.65 m above the membrane surface. Overflow conditions at the storage tank guaranteed constant transmembrane pressure. Filter holders, silicon tubes and glass bottles for collection of filtrate water were autoclaved prior to experiment. Ultrafiltration membranes have been soaked in nanopure water (Bearnstead, Thermo Scientific, Basel, Switzerland) for 24 h before starting the experiment. The six membrane systems were split into three different treatments, each with two replicates (Figure 1): the control treatment (referred to as CON) received lake water only. A Microcystis bloom was simulated in two replicates: the lake water was enriched with the cyanobacterium Microcystis aeruginosa PCC 7806 (about 2×10^8 cells) once every 24 h (treatment subsequently referred to as LMA: living Microcystis aeruginosa). A collapsing cyanobacterial bloom was simulated in the last treatment. A culture of Microcystis aeruginosa PCC 7806 was first frozen for 3 h at −20°C, and thawed before adding to the membrane system once every 24 h (referred to as DMA: dead Microcystis aeruginosa). The cyanobacterial cells were directly added above the filtration membrane module to avoid MC degradation processes in the storage tank or during tubing passages. All filter systems were kept in the dark. The filtrate water of all treatments was collected every 24 h, and the volume was determined to quantify the permeate flux (as L m^{-2} h^{-1}). A subsample of 1 mL of each filtrate was fixed with 50 µl glutaraldehyde (2.5% final concentration) for cell enumeration at the flow cytometer. The rest was stored at 4°C for microcystin (MC) quantification (usually done within 24 to 48 h). The experiment was running for 21 days. At the end of the experiment, the ultrafiltration membranes were cut into three equal parts that were subjected to the following analyses: (i) One part of the filter was used to quantify MCs by HPLC that possibly remained in or attached to the biofilm on the filters. (ii) Phylogenetic analyses (454 tag pyrosequencing) were performed with the biofilm on another filter part, and (iii) the last part was used to take a closer look on biofilm structures by non-invasive methods such as Optical Coherence Tomography (OCT) (model 930 nm Spectral Domain, Thorlabs GmbH, Dachau, Germany). OCT images were analysed for average thickness and relative roughness using image analysis software developed under Matlab (MathWorks, Natick, US) [12]. To determine the exact membrane area of the pieces image analysis (Zeiss, AxioVision 4.7) was applied using a microscope (AxioImager.Z1, 1 x EC Plan-Neofluar, Zeiss) and a CCD camera (AxioCam MRm, 12 bit grayscale, 1388×1040 px, Zeiss).

Flow cytometric enumeration of cells in the filtrate water

Samples for flow cytometry were stained with DAPI (4′,6-diamidino-2-phenylindole, 1 µg mL^{-1} final concentration) for 15 min in the dark. Subsequently, samples were analysed using an Influx V-GS cell sorter (Becton Dickinson, Inc., San Jose, CA) equipped with a UV laser (60 mW, 355 nm; CY-PS; Lightwave Electronics) for detection of DAPI fluorescence, and a blue laser (200 mW, 488 nm; Sapphire; Coherent Inc.) for scattered light

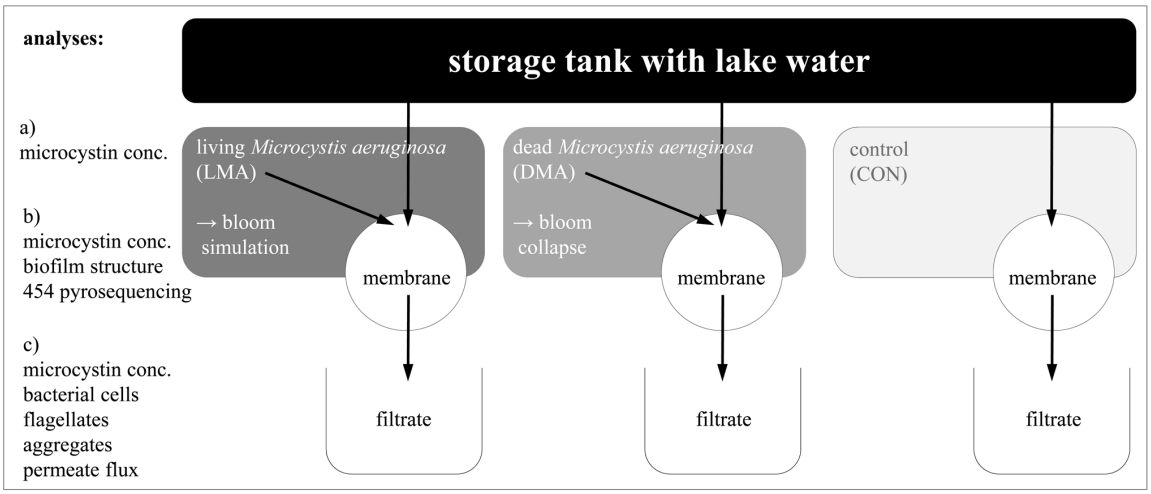

Figure 1. Schematic view of the gravity-driven membrane (GDM) system. Depicted are the three different operating treatments (LMA, DMA, and CON), the ultrafiltration membrane (150 kDa) and the filtrate collection. Analyses that were carried out during this study a) in the feed water, b) in/on the biofilm, and c) in the filtrate are listed on the left side of this overview.
doi:10.1371/journal.pone.0111794.g001

and autofluorescence of flagellates. If necessary, and to avoid particle coincidence, samples were diluted with sheath fluid (2.5 g L^{-1} NaCl; filtered by 0.2 µm-pore-size). Sample volume was calculated from the analysed sample weight. Data obtained by flow cytometry were analysed with the custom-made software ViiGate 1.0a. Bacterial cells were identified using side scattered light (SSC) versus DAPI fluorescence (431 nm), flagellates were determined on the basis of SSC versus green fluorescence (531 nm). Bacterial aggregates were operationally defined by their DAPI fluorescence and scatter properties equal to or higher than that of flagellates [22]. Cyanobacterial cells of the *Microcystis aeruginosa* PCC 7806 culture were identified using SSC versus their auto-fluorescence at 692 nm (without prior staining).

Extraction of microcystins

The entire filtrate was collected for solid phase extraction of MCs. The C18 cartridges (1 g, 60 mL, Mega Bond Elute, Varian, Agilent Technologies, Basel, Switzerland) were first equilibrated with 10% MeOH before adding the filtrate water. Afterwards, the MCs were eluted with 100% methanol. The samples were dried in a vacuum rotary evaporator at 40°C and 35 mbar. The residues were re-suspended in 1 ml 60% MeOH, the microcystin quantification was performed by HPLC in duplet. The MC concentrations in the filtrate were always measured 24 h after injection and were expressed as percentage of the MCs that were removed from the system (removal efficiency) or as MC removal rates (ug L^{-1} d^{-1}).

At the end of the experiment, GDM polyethersulfone membranes were first frozen at −23°C for 3 h to isolate the MCs from the biofilm. After thawing, the biofilms were extracted twice with 5 mL 60% MeOH for 1 h, and both extracts were combined. Accordingly, the solvent was evaporated (40°C and 35 mbar), and the samples were prepared for HPLC analysis. The residues were re-suspended in 1 ml 60% MeOH, and centrifuged for 5 min at 10′000 rpm. Afterwards, the supernatants were taken for microcystin quantification by HPLC (as described above).

454 tag pyrosequencing analysis

Prior to the analysis, the filter parts of both replicates were pooled. The DNA extraction of the biofilm bacteria was performed using the UltraClean Water DNA isolation kit (MO BIO Laboratories, Inc.). Subsamples of 300 µl of DNA suspension (final concentration 4–10 ng μL^{-1}) of all three DNA extractions (CON, LMA, and DMA) were sent to Research and Testing Laboratory, Inc. (Lubbock, TX, USA) for further processing. Partial 16S rRNA gene encoding sequences were obtained from 454 pyrosequencing (Roche FLX platform) following Assay b.9 by using the primer pair 799F and 1115R that exclusively amplify DNA of heterotrophic bacteria and exclude cyanobacteria from the process [23]. Raw data (68′981 Reads; mean raw read length 378.4 base pairs) were processed by a custom-made pipeline on a local computer cluster consisting of 16 units (each equipped with an 8 core AMD FX-8150 CPU, 16 GB RAM and a 128 GB SSD hard disk) and a separate control workstation. The program was developed in DELPHI and run under Windows 7. The processing of the raw data is extensively described elsewhere [24]. In brief, reads were denoised at the level of flowgrams according to Quince and co-workers [25]. Afterwards, quality filtering strategies were applied to finally end up with the number of 27′391 sequences (raw reads reduced by 60%) corresponding to 956 operational taxonomic units (OTUs, 3% similarity). A distance matrix was calculated and the OTUs were produced after the pairwise alignment (Needleman-Wunsch algorithm) by average linkage at similarity levels of 97%. OTUs were assigned to taxonomic entities on the level of similarity of the OTU to the most closely related sequence in the SILVA reference data base (release 109) [26]. OTUs were grouped into different levels of sequence identity: ≤ 3% divergence in 16S rRNA gene sequence corresponds to species level, ≤5% to genus level, and ≤10% to family level. Finally, the OTUs of the both treatments were compared with each other. Only OTUs were included that were >0.5% of sequences per sample (CON or LMA+DMA) and that occurred at least ten times more frequently in one treatment than the other. Shannon's diversity index was estimated using the formula $H' = -\Sigma(P_i * \ln P_i)$, where P_i is the relative abundance of the sequences per sample. The index H′ is used to characterize the diversity of species or species-like units (OTUs) in a community.

Results

Physical parameters: Permeate Flux and structure of the biofilms

Flux stabilization was observed approximately after eight to ten days of the experiment in all three treatments albeit great differences between the control and both Microcystis treatments (Figure 2). A mean flux of 4.7 L m^{-2} h^{-1} was measured after 12 days in the CON treatment. In one of the two replicates, the flux stayed constant until the end of the experiment. In the second replicate, the flux increased slowly to 6.9 L m^{-2} h^{-1} on day 21. Accordingly, the mean thickness (as assessed by OTC measurements) of the biofilms of both control replicates at the end of the experiment were slightly different. The biofilm of the first replicate had a thickness of about 125 (±23) μm; the biofilm of the second replicate was 96 (±17) μm thick. The second replicate was less heterogeneous than the first replicate, but both exhibited low relative roughness values of 0.49 and 0.35, respectively. The permeate flux in both Microcystis treatments showed a similar trend. Stabilization could be observed at a mean flux of 1.6 L m^{-2} h^{-1} in the LMA and of 2.0 L m^{-2} h^{-1} in the DMA treatment. In both treatments, mean permeate flux decreased further to 1.0 L m^{-2} h^{-1} and 1.36 L m^{-2} h^{-1}, as measured at the end of the experiment. Thus, the mean flux in the Microcystis replicates was about 80% lower than in the CON treatment. Biofilms in the DMA treatment were about six to seven times thicker as in the CON treatment with values of 625 (±33) μm and 796 (±29) μm for both replicates. Unfortunately, a quantification of the biofilm thickness in the LMA treatment could not be carried out.

Microcystin removal

The microcystin removal efficiency of the biofilms was calculated from the amount of MCs that was injected via living or dead Microcystis cells into the systems and the amount that was measured 24 hours later in the filtrates (Figure 3, upper panel). In the LMA treatment, both replicates were working similarly, showing already high removal efficiency of almost 70% at the beginning. After 10 days of the experiment, the biofilms of both replicates showed nearly 100% removal efficiency; complete removal was achieved after 15 days and remained constant until the end of the experiment. Both replicates of the DMA treatment started with a low removal efficiency of about 10% during the first three days of the experiment. One of the replicates developed a biofilm that was able to remove the MCs completely after 15 days. The MC removal efficiency of the biofilm of the second replicate decreased again to less than 80% (Figure 3, upper panel). The MC removal rates in both treatments increased during the course of the experiment up to 440, respectively 300 μg L^{-1} d^{-1} (Figure 3, lower panel).

Altogether, 4.6×10^9 living or dead cells of M. aeruginosa (containing 262.5 μg MCs) were added to each MC-replicate of the GDM systems throughout the duration of the experiment (Table 1). About 10% (27 μg) of the added MCs were found again in the filtrates of each replicate of the LMA treatments during the entire experiment, 96.5 μg (36.8%) were found on the LMA filters at the end of the experiment. Thus, 140 μg of MCs were removed in both replicates during the course of the experiment. Only 0.5 μg MC was found on the filter in the DMA treatments, 99 μg, respectively 121 μg have been collected in the DMA filtrates of both replicates; 141–163 μg of the MCs were degraded during the course of the experiment.

Cell numbers

Bacterial single cell numbers in the CON filtrates constantly increased during the course of the experiment (Figure 4, upper panel) up to 0.1 and 0.3×10^6 cells mL^{-1}, respectively. Similarly, flagellate and bacterial aggregate numbers increased slowly, but stayed on comparable low mean levels of 280 flagellates mL^{-1} and 78 aggregates mL^{-1} on day 21 of the experiment (Figure 4). In contrast, both MC treatments showed higher cell numbers as

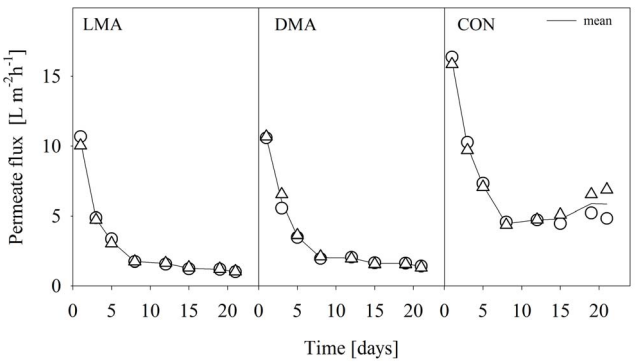

Figure 2. Evolution of the permeate flux. The flux is shown in L m^{-2} h^{-1} for the filtration of differently treated feed water sources (LMA, DMA, and CON according to Figure 1). The two replicates per system are shown as circles and triangles, and are connected by the mean.
doi:10.1371/journal.pone.0111794.g002

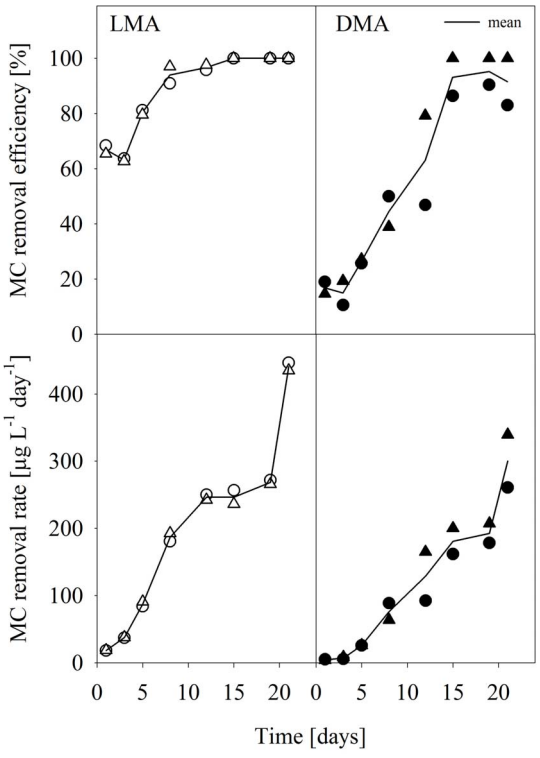

Figure 3. Microcystin removal and removal rates. Time course of the microcystins (MCs) removal efficiency of the GDM system (upper panel) and the MC removal rate (lower panel) in the two *Microcystis* treatments (LMA and DMA according to Figure 1). The two replicates per system are shown as circles and triangles, and are connected by the mean.
doi:10.1371/journal.pone.0111794.g003

Table 1. Microcystin concentrations.

	LMA		DMA	
	Replicate 1	**Replicate 2**	**Replicate 1**	**Replicate 2**
Total amount of MCs injected over 21 days	262.5 (100)	262.5 (100)	262.5 (100)	262.5 (100)
Amount of MCs in the filtrate on day 21	0 (0)	0 (0)	0 (0)	2.9 (1.11)
MCs on the filter at the end of the experiment (on day 21)	96.5 (36.8)	96.5 (36.8)	0.5 (0.2)	0.5 (0.2)
Total amount of MCs in the filtrates, collected for 21 days	28 (10.6)	26 (9.9)	99 (37.7)	121 (46.1)
MCs loss	138 (52.6)	140 (53.3)	163 (62.1)	141 (53.7)
Threshold concentration of less than 1 μg L^{-1} was reached on day	15	15	15	Not reached

Overview about the amount of MCs [μg; (%)] totally injected, found on the biofilm or in the filtrate, and the estimated loss of MCs during the experiment. Last row shows the day, at which the threshold concentration of less than 1 μg L^{-1} was reached (LMA and DMA according to Figure 1).
doi:10.1371/journal.pone.0111794.t001

compared to the CON treatment. Between 0.8 and 0.5×10^6 bacterial single cells mL^{-1} were found in both replicates of the LMA treatment at the end of the experiment. Comparable numbers of bacterial single cell numbers were determined also in the replicate 1 of the DMA treatment (0.4×10^6 mL^{-1}). The bacterial single cell numbers in the corresponding second replicate of the DMA treatment were about ten times higher (3.9×10^6 mL^{-1}). Flagellate numbers in the filtrates of the LMA treatment were comparable high at the end of the experiment (mean value 0.67×10^4 mL^{-1}), as well as bacterial aggregates (mean value 0.8×10^3 mL^{-1}). Comparable numbers of flagellates were found in the filtrate of the first replicate of the DMA treatment (0.54×10^4 mL^{-1}) as well as the highest amount of aggregates (4.0×10^3 mL^{-1}) at the end of the experiment. However, rather low numbers of flagellates and aggregates were found in the second replicate of the DMA treatment, 0.15×10^4 mL^{-1} and 0.1×10^3 mL^{-1}, respectively (Figure 4).

Phylogenetic analyses

A total of 27′391 sequences, assigned to 956 OTUs, were evaluated after removing low quality reads and chimeric sequences. For the bacterial communities of the MC-treated biofilms, about 452 OTUs (8′064 sequences; LMA) and 378 OTUs (8′048 sequences; DMA) were determined (Figure 5A); slightly more sequences were obtained for the CON bacterial community (11′279 sequences; 551 OTUs). The bacterial communities of the three biofilms shared only 11% of all OTUs (105 OTUs), but comprised 54% of all sequences (Figure 5A and 5B). The OTUs therein were large, and consisted of 140 sequences on average. This core community consisted predominantly of Sphingobacteriales (Bacteroidetes; 48% of the shared OTUs) and Comamonadaceae (Betaproteobacteria, 45% of the shared OTUs). The CON treatment consisted of the largest amount of OTUs (35% of all OTUs) that were unique to this special treatment, but comprised only 1764 sequences (5.2 sequences per OTU). LMA and DMA shared 108 OTUs, the average sizes of these OTUs were about 50 sequences per OTU (Figure 5A and 5B).

Most of the sequences in the combined LMA and DMA (MA) communities received taxonomic assignments at least to the genus level (93% in the LMA treatment, 96% in the DMA treatment), affiliation to families could be determined for 97%, respectively 99% of these sequences. However, only 44% of all sequences in the CON communities were assigned to the genus level, and 52% to the family level. With a high divergence in similarity of more than 12% to the closest known relative, two large OTUs were assigned to either Myxococcales (1′990 sequences) or to Fibrobacteres (2′357 sequences).

Bacterial taxa favoured by Microcystis addition

Addition of Microcystis cells led to compositional differentiation between the communities of both MC-treated and the CON biofilms. Over one third (38.3%) of all sequences in the CON assemblage was affiliated with Deltaproteobacteria, a class that was significantly less abundant in the combined LMA and DMA (MA) communities (5.0%) (Figure 6). The Fibrobacteres (36.6%) and the Alphaproteobacteria (19.4%) were the second and third most abundant taxa affiliated with the CON assemblage, both taxa were underrepresented in MA assemblage as well. These three classes comprised almost 95% of all sequences in the CON assemblage. However, more than one third (40.4%) of all sequences in the MA assemblage was affiliated with Betaproteobacteria that were found only marginally in the CON communities. Firmicutes (22.4%) and Gammaproteobacteria (12.8%) were the second and third most abundant taxa in the MA assemblage, but were not found in the communities of the CON treatment. These three classes comprised 75.6% of all sequences. Additionally, minor fractions of Candidate division TM7 and Bacteroidetes were present in both assemblages, Spirochaetes only to a minor extent in the MA assemblage (1.3%).

Most of the bacterial genera found in the CON assemblage were typically isolated from microbial biofilms in drinking or freshwater reservoirs or pipelines, such as Hirschia, Phenylobacterium, or Comamonas. Haliangium or the Myxococcales were present in anaerobic filter sediments or suboxic freshwater ponds (Table S1 in File S1). The Fibrobacteres were isolated before from upper sediment layers and biofilm samples. Bacterial sequences affiliated with Sphingomonas containing MC degradation proteins were only found in the CON assemblage, where they made up 12% of all sequences (Table S1 in File S1). Other genera of the Alphaproteobacteria that contain MC degradation proteins or MC dependent proteins have been found only in the MA assemblage, such as Azospirillum or Magnetospirillum, as well as the Rheinheimera (belonging to the Gammaproteobacteria) or Spirochaetales (Table S2 in File S1). Some other bacterial genera have been repeatedly found in the Planktothrix layer of Lake Zurich, such as Variovorax (5.4% of all sequences in the MA assemblage) or unknown genera belonging to the Sphingobacteriales (6.5%). Paucibacter known to degrade MCs has been only found in the MA assemblage (3.2%). The Firmicutes (22.4% of all sequences of the MA assemblage) comprised mainly of Acidaminobacter that are typical inhabitants of suboxic freshwater ponds.

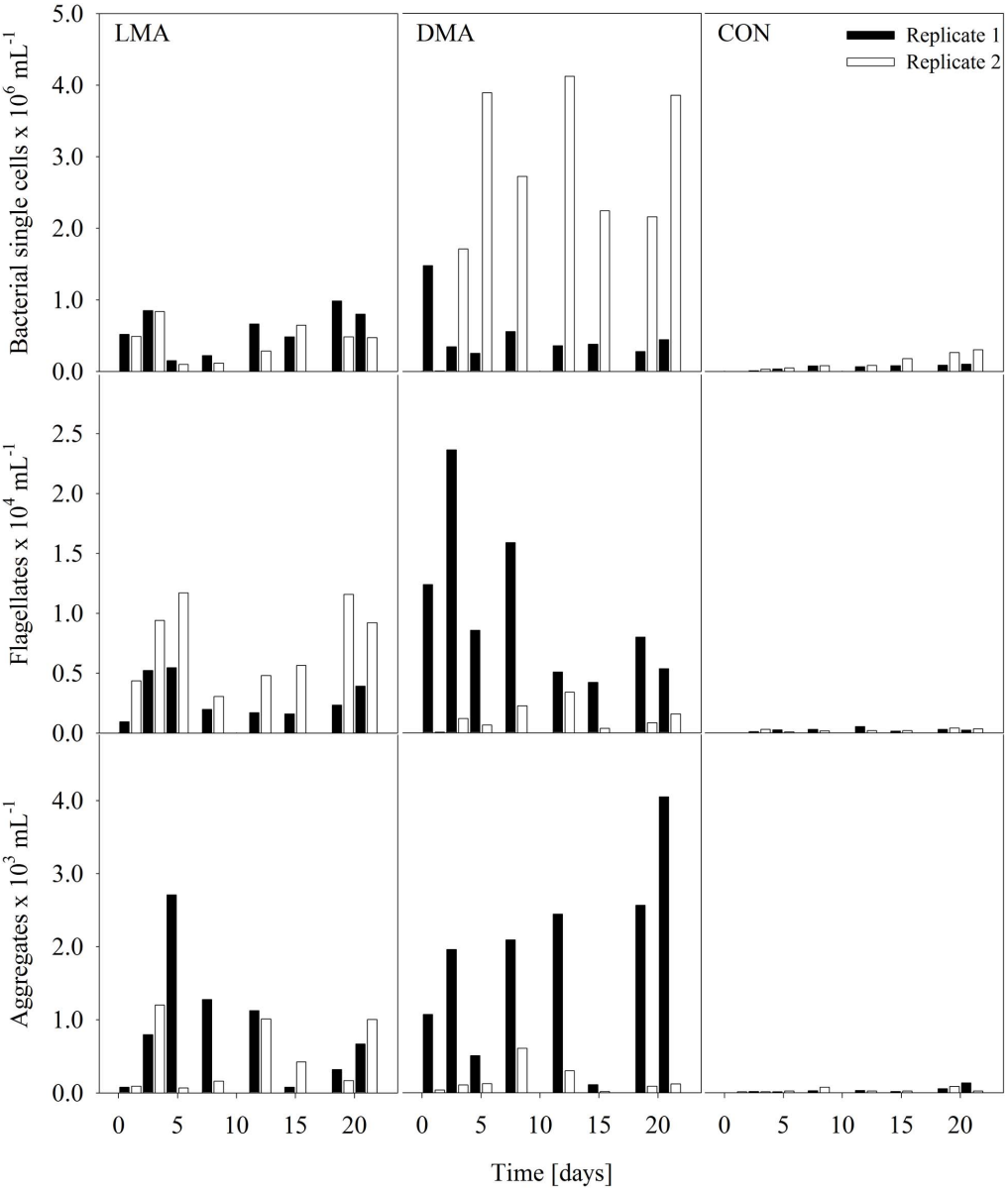

Figure 4. Cell abundances in the filtrate. Regrowth of bacterial single cells (upper panel), flagellates (middle panel) and flagellate-inedible bacterial aggregates (lower panel) in the filtrate of the three different treatments (LMA, DMA, and CON according to Figure 1).
doi:10.1371/journal.pone.0111794.g004

Discussion

Performance of the GDM system and fate of microcystins

Two factors have a strong impact on the performance of GDM systems: the composition of the microbial community (bacteria and predators) and the organic carbon content of the feed water [12]. In the latter study, the almost complete absence of metazoan organisms resulted in smooth and homogeneous biofilm structures. In our study, this was indicated by the comparably low values of relative roughness of both CON replicates. In the absence of predation, the total organic carbon (TOC) content governs the permeability of the biofilms. The decrease of the permeate flux with increasing TOC content is due to, both, a higher accumulation of particulate matter (originated from the influent) and a higher bacterial growth because of the higher nutrient loads upon cell lysis [12]. Cyanobacterial blooms are such a source of high TOC. The enormous load of cyanobacterial biomass in both Microcystis treatments resulted in the massive increase in biofilm thickness. Thus, both Microcystis treatments lost 80% of their performance, which was shown by a much lower flux and permeate, respectively.

At the same time, these biofilms were able to degrade MCs. A complete removal of the MCs by the biofilms in our study could be achieved after 15 days, which was one week after the stabilization of the flux, i.e., after the development of a stable biofilm. It is conceivable that after that time bacteria capable of the degradation of MCs had accumulated on the biofilm. The key role of the bacterial biofilm in MC degradation can be confirmed by comparing the performance of the GDM system during the initial phase of the experiment and after flux stabilization. A reduction of 10% during the first three days of the experiment (Figure 3) cannot be attributed to the not yet developed biofilm but rather to

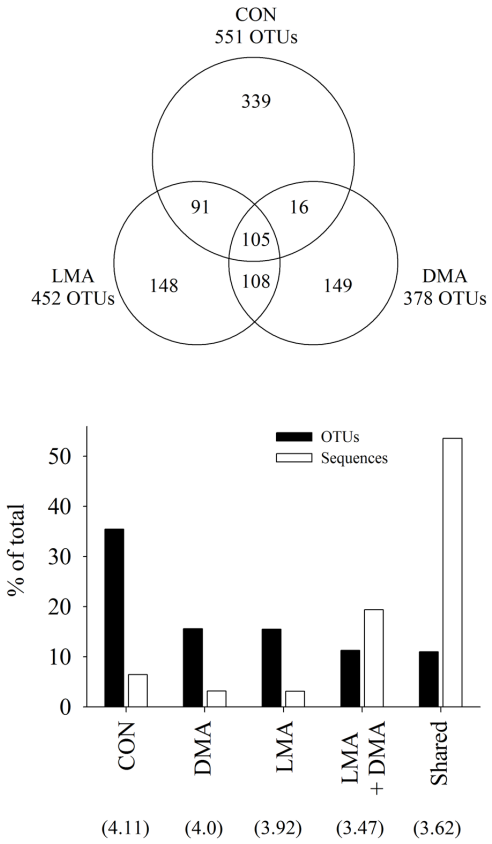

Figure 5. Overview of microbial diversity analysis. Upper panel: Venn diagram of shared OTUs among the biofilms of the three treatments. Lower Panel: percentage of operational taxonomic units (OTUs) and sequences found in the different treatments. Number in brackets marks the Shannon's diversity index (*H'*) for each treatment (LMA, DMA, and CON according to Figure 1).
doi:10.1371/journal.pone.0111794.g005

adsorption processes, either to the filtration units or to cyanobacterial cells that retained on the filter membrane.

However, the potential of biofilms to remove MCs does not only depend on the presence and fast accumulation of possible MC degrading bacteria, but also on their efficiencies to biodegrade these MCs. Degradation rates of various bacterial strains might range between 1.5 up to several 10′000 $\mu g\ L^{-1}\ d^{-1}$ [16] Already after 15 days, the added MCs were completely removed. In our study, MC removal rates increased constantly after a short lag phase, but did not reach a plateau. Therefore, the degradation capability of the aged biofilm might have further increased and even higher concentrations of MCs might be successfully degraded. The previous natural exposure of the feed water to cyanobacteria and MCs (as is the case for water from Lake Zurich) seems to be a significant driver for a fast performance of a MC degrading biofilm and thus its accelerated maturation [27]. Lake Zurich contains large populations of Planktothrix rubescens, a filamentous microcystin-producing cyanobacterium, which is frequently found in natural pre-alpine lakes [2]. It should be noted that the biofilm in our study was selectively grown under Microcystis bloom conditions. It remains to be investigated, how a bacterial biofilm without prior presence of MCs would respond to Microcystis addition. Another interesting aspect, which was beyond the scope of our experiment, would be the comparison of the bacterial composition in the biofilms by comparing MC-producing with MC-non-producing strains. This would allow for a more specific assessment, which shifts in microbial community compositions were caused by other substrates provided by the addition of cyanobacterial biomass.

Drinking water quality

Pro- and eukaryotic microbes can regrow during drinking water treatment and the distribution of non-chlorinated potable water [28]. The total bacterial cell counts in tap and mineral water may reach values between 1.5×10^5 to 5×10^5 cells mL^{-1} [29,30]. In our study, bacterial abundances in the CON treatment were within this range. Bacteria most likely regrew in biofilms underneath the filter membranes or on materials downstream the filtration process and dropped into the filtrate [29]. Inadequate disinfection, hydraulic retention time, flow regime, pipe material, temperature, source of water or corrosion could lead to the development of a microbial biofilm. However, excessive regrowth

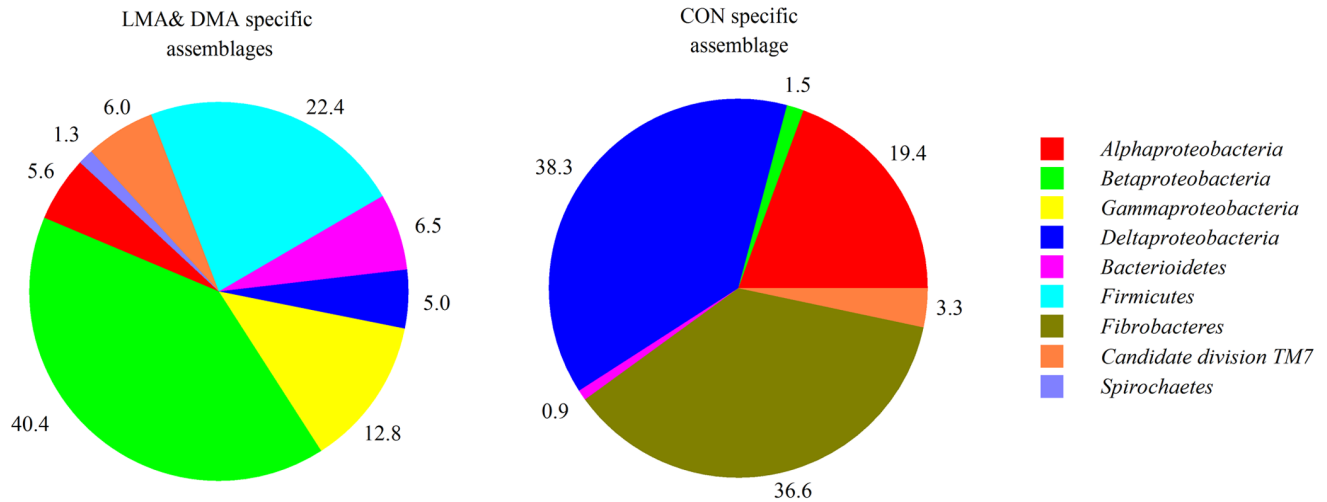

Figure 6. Phylogenetic composition of bacterial biofilms. Phylogenetic composition [%] of bacteria specific for the (combined) LMA and DMA assemblage (left) and for the CON (right) assemblage. Specificity for either assemblage was defined by the size of the OTU (>0.5% of sequences per sample) and by a ten times higher occurrence in one category than the other (LMA, DMA, and CON according to Figure 1).
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in drinking water supply systems can be also triggered by nutrient introduction. It is conceivable that the massive load of cyanobacterial biomass in our study led to higher bacterial cell numbers, not only under the membrane filter but also in the filtrate. The increased availability of various substrates released from broken cells in the DMA treatment resulted in even higher cell numbers in the corresponding filtrates. As the drinking water quality may be deteriorated by the presence of pathogenic bacteria, an adequate storage of the drinking water is important to avoid re-contamination [31]. The focus in our study was on the removal of cyanobacterial toxins. However, the effect of other components of cyanobacterial cells that enhance regrowth remains to be investigated.

Drinking water storage or distribution systems represent functional ecosystems with well-established and structured microbial communities [32]. The increasing numbers of bacteria support the succession of protists: on average 10^5 cell L^{-1} were found in the water phase and 10^3 cells cm^{-2} in different biofilms [32]. As a result, some bacteria may aggregate to overcome the predation pressure by protists as it was shown in field and laboratory studies before [15]. The regrowth of bacteria underneath the filter membranes or on materials downstream the filtration process of the LMA treatments in our study stimulated the growth of bacterivorous flagellates, followed by an increase in bacterial aggregates (Figure 4). Interestingly, the DMA replicates showed two different responses: high numbers of free-living planktonic bacteria were established in the absence of flagellates in one replicate of the DMA treatment, whereas the numbers of free-living bacteria in the second replicate were low, as bacteria either were grazed by flagellates or formed aggregates in order to resist predation. This illustrates that there might be variability in the primary microbial assemblages of such filtrates that may have consequences for the development of drinking water quality.

Differences in microbial biofilms

Comparative phylogenetic analyses of 16S rDNA has increased our understanding of microbial diversity in environmental samples, since only few of the identifiable major phyla within the domain Bacteria have cultivable representatives [33]. Many of these uncultivated bacteria are found in diverse habitats in extraordinarily high abundances and might be at best only remotely related to strains that have been characterized by phenotype or by genome sequencing [33]. This in turn implies that there is very limited understanding of their respective physiologies, e.g., their substrate degradation potential. Calculating distances to sequences in a well-curated database [26] allowed us to classify OTUs to the closest taxonomic level. The CON treatment was the most diverse of all treatments, as reflected by the high number of small OTUs and an H′ index of 4.11. With the exception of river and stream habitats [34] biofilms that occur under more oligotrophic conditions (as in the CON treatment) seem to be rather understudied. As a consequence, more "exotic" species were found that had a high distance to the closest known relatives (CKR), such as uncultured Myxococcales (11.3–12.1% distance to CKR) and Fibrobacteres (12.7% distance to CKR) (Table S1 in File S1).

The massive load of cyanobacterial biomass selected for a few large OTUs (H′ = 3.47 of the combined MA) and the bacterial taxa represented by these OTUs were from a more "known" bacterial diversity (Table S2 in File S1). An intriguing example for typical freshwater bacteria that were present in our MA samples is a set of OTUs with <3% distance to known culturable genera, such as Azospirillum, Pelomonas, or Undibacterium (Table S2 in File S1). Moreover, oxygen subsaturation in the thick biofilms of the MA treatment was suggested by the presence of obligate anaerobic bacteria such as Desulfovibrio, Acidaminobacter, Fusibacter, or Spirochaetales. The ability to remove MCs appears to be not only common for aerobic bacteria, but can also be a feature of anaerobic microorganisms (not otherwise specified) that were found in lake sediments or sediments of water recharge facilities [35]. It seems that several Spirochaeta contain the MC-LR degradation protein MlrC (Table S2 in File S1). However, further studies are needed to determine possible MC degrading bacteria in anoxic environments. Interestingly, Bacteroidetes such as Sphingobacteriales that are common in the Planktothrix layer of Lake Zurich [36] were also present in high proportions in the MA treatments only. The natural co-occurrence of these bacteria with MC-producing cyanobacteria might indicate a possible and so far unknown role in the MC-degradation process.

Previous investigations have suggested the Sphingomonadales (Alphaproteobacteria) being the major MC degraders in aquatic environments: genetic studies on Sphingomonadaceae revealed the distinct gene cluster mlrABCD to be involved in MC removal [37], because it encodes for an enzymatic ring cleavage and thus a linearization of the MCs. However, during a massive cyanobacterial bloom in Lake Erie only ~1% of the total bacterial community could be attributed to Sphingomonadales [38] and also the metagenomic identification of bacterioplankton taxa involved in MC degradation revealed only a minor importance of these bacteria [20]. Our data also suggest that Sphingomonadales may not necessarily be relevant in MC degradation, as they only enriched in the CON treatment (Figure 6, Table S1 in File S1).

Our data indicate that Betaproteobacteria may be more important amongst the major MC degradation bacteria as they constituted the major fraction (>40%) in the MA biofilms but were hardly found in the CON assemblages. OTUs were found in high quantities that were closely related to Paucibacter, capable of degrading MCs [39] and Variovorax, containing MC-LR degradation proteins, (Table S2 in File S1). The importance of Betaproteobacteria (mainly Burkholderiales and Methylophilales) has already been suggested before based on laboratory microcosms experiments amended with MCs [38]. Interestingly, recent studies revealed that Betaproteobacteria such as Methylophilales were capable of degrading MCs but lacked the mlr cluster, thereby providing an alternative and so far unknown means of MC removal [20]. Studies focussing on the detection of the mlrA gene as the only marker for MC degradation bacteria might therefore underestimated the possible presence of other MC degrading bacterial taxa [16,20,21].

Conclusion

i. We demonstrated that GDM ultrafiltration systems provide a fast and efficient way to remove MCs from drinking water. However, it should be noted that complete MC degradation only took place one week after establishment of a stable biofilm.

ii. Addition of live or dead Microcystis cells led to remarkable differences between the bacterial communities of both MC-treated and the CON biofilms.

iii. Betaproteobacteria were identified as potentially important taxa for MC degradation in the MA biofilms. Additionally, Spirochaeta and Bacteroidetes such as Sphingobacteriales were enriched in these biofilms, and might indicate their so far unknown role in the MC degradation process.

Supporting Information

File S1 File S1 contains two supplemental tables: **Table S1, Phylogenetic composition.** Affiliation of bacteria in the CON assemblage (OTUs specific for CON treatment), number of OTUs and sequences, and phylogenetic distances of OTUs and associated sequences in the CON assemblage to the most closely related genotype in the SILVA reference database. **Table S2, Phylogenetic composition.** Affiliation of bacteria in the LMA & DMA assemblage (OTUs specific for the *Microcystis* treatment), number of OTUs and sequences, and phylogenetic distances of OTUs and associated sequences in the LMA & DMA assemblage to the most closely related genotype in the SILVA reference database.

(DOCX)

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Author Contributions

Conceived and designed the experiments: EK TP ND EM JP JFB. Performed the experiments: EK JV TP ND TS. Analyzed the data: EK JV TP ND EM JP JFB. Contributed reagents/materials/analysis tools: EM JP. Contributed to the writing of the manuscript: EK TP ND EM JP JFB.

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CHAPTER 2 - STRUCTURE ELUCIDATION AND ENZYMATIC CHARACTERIZATION

MANUSCRIPT II

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The toxicity and enzyme activity of a chlorine and sulfate containing aeruginosin isolated from a non-microcystin-producing *Planktothrix* strain

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ABSTRACT

The toxicity of six different *Planktothrix* strains was examined in acute toxicity assays with the crustacean *Thamnocephalus platyurus*. The presence of toxicity in two strains could be explained by the occurrence of microcystins. The other four *Planktothrix* strains were not able to produce microcystins due to different mutations in the microcystin synthetase (*mcy*) gene cluster. In these strains, toxicity was attributed to the presence of chlorine and sulfate containing compounds. The main representative, called aeruginosin 828A, of such a compound in the *Planktothrix* strain 91/1 was isolated, and structure elucidation by 2D NMR and MS methods revealed the presence of phenyllactic acid (*Pla*), chloroleucine (*Cleu*), 2-carboxy-6-(4′-sulfo-xylosyl)-octahydroindole (*Choi*), and 3-aminoethyl-1-*N*-amidino-Δ-3-pyrroline (*Aeap*) residues. Aeruginosin 828A was found to be toxic for *T. platyurus* with a LC_{50} value of 22.4 μM, which is only slightly higher than the toxicity found for microcystins. Additionally, very potent inhibition values for thrombin (IC_{50} = 21.8 nM) and for trypsin (IC_{50} = 112 nM) have been determined for aeruginosin 828A. These data support the hypothesis that aeruginosins containing chlorine and sulfate groups, which were found in microcystin-deficient *Planktothrix* strains, can be considered as another class of toxins.

1. Introduction

Climate change as well as the anthropogenic input of nutrients into freshwaters has resulted in an increase of cyanobacterial biomasses in many lakes and rivers throughout the world during the last decades (Paerl and Huisman, 2008). These organisms are often found to accumulate in very high densities also in lakes that are intensively used as recreational areas or as drinking water reservoirs (Posch et al., 2012). However, cyanobacteria are known to produce and store intracellularly a wide variety of bioactive secondary metabolites, and a collapse of such a bloom might liberate high amounts of these compounds into the water. In recent years, substantial progress has been made to identify possible harmful compounds from different cyanobacterial genera. Most of these substances could be assigned to distinct chemical oligopeptide classes, e.g. microcystins, cyanopeptolins, anabaenopeptins, aeruginosins, cyclamids, microginins and microviridins (Welker and von Döhren, 2006). These peptides exhibit various biological activities such as the inhibition of different phosphatases and proteases (Welker and von Döhren, 2006). Microcystins (MCs) have been found to be responsible for countless animal poisonings all over the world, and even for human toxicity (Sivonen and Jones, 1999), and cyanopeptolins and aerucyclamides received some attention due to their toxicity to *Thamnocephalus platyurus* (Blom et al., 2003; Gademann et al., 2010; Portmann et al., 2008a, 2008b).

There is intense discussion about the possible ecological role of MCs in the environment; however, MCs are still regarded to be the primary defence mechanism of cyanobacteria against grazers (Blom et al., 2001; Kurmayer and Jüttner, 1999). MC-deficient *Planktothrix* genotypes are typically found in rather low proportions of the total cyanobacterial population (Ostermaier and Kurmayer, 2009), but might occasionally exceed 50% of the total abundances. Reasons for the lack of MC production might be various point mutations (e.g. insertions or deletions) or the loss of genes encoding the MC synthetase (*mcy*) gene cluster (Christiansen et al., 2006, 2008). However, the loss or dysfunction of the *mcy*

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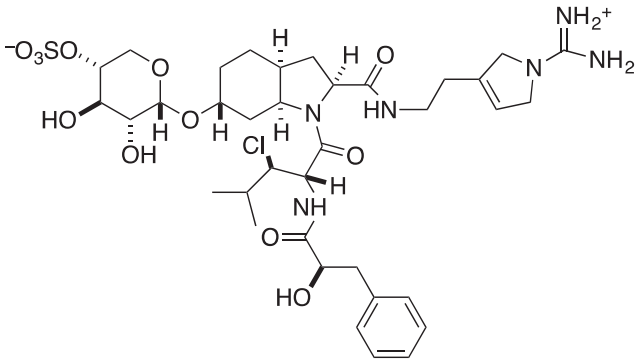
gene cluster did not seem to be disadvantageous for the overall success of the *Planktothrix* genotypes. This is supported by the linear relation of MC deficient genotypes to the total population density of bloom forming *Planktothrix* populations in European lakes (Ostermaier and Kurmayer, 2009). In order to explain these findings it was suggested that an alternative peptide or peptide class might functionally compensate for the lack of MCs. Here we report on a peptide class, the chlorine and sulfate containing aeruginosins, which were found in MC-deficient strains, and which exhibited acute toxicity against the crustacean *Thamnocephalus platyurus*.

2. Material and methods

2.1. General experimental procedures

^{1}H NMR spectra were recorded on a Bruker Avance III 600 MHz with a 5 mm BBFO plus probe or a Bruker Avance III Ascend 700 MHz spectrometer with a 5 mm TCI (H-C/N-D) cryo probe at room temperature. Chemical shifts (δ-values) are reported in ppm, spectra were calibrated related to the solvent residual proton chemical shift (DMSO, $\delta = 2.50$). The coupling constants are specified in Hz. HRMS spectra were recorded on a Bruker maXis 4G instrument. Identification and purification of aeruginosin 828A (**1**) was performed on a Shimadzu 10AVP HPLC system equipped with an automated sample injector, a thermostated column compartment, and photo diode array detector. Mass spectra were recorded on a combined LC–MS (LCQ Duo mass spectrometer, Finningan Thermoquest, USA) equipped with an electrospray ionization source (ESI-MS).



Aeruginosin 828A (**1**)

2.2. Culture, extraction, and bioassay-guided fractionation

A culture collection of six *Planktothrix* strains was established in the laboratory at the beginning of this study. All cyanobacteria were grown in 300 mL Erlenmeyer flasks at 20 °C under constant light conditions at an irradiation of 6 μmol m^{-2} s^{-1} from fluorescent tubes (Osram 930; Lumilux Delux; Warm White 3000K) in 120 mL mineral medium described by Jüttner et al. (1983). Two of the six *Planktothrix* strains were capable of producing MCs, four strains lacked the MC production either due to point mutations or complete loss of the *mcy* gene cluster (summarized in Table S1, Supplementary material). The analysis of the *mcy* gene cluster in *Planktothrix* strains was carried out as part of another study as described elsewhere (Christiansen et al., 2008).

Frozen biomass of all six *Planktothrix* strains was extracted twice with 50% MeOH (10 mL per gram of wet cell biomass) for 2 h in the dark. After centrifugation (25,700 × *g* for 15 min) the supernatants (crude extracts) were fractionated by HPLC equipped using a reversed phase column (Hydrosphere C18, YMC, 4.6 × 250 mm, Stagroma, Reinach, CH) using two solvents: UV-treated deionised water and acetonitrile. The solvents were free of trifluoroacetic acid (TFA) to avoid undesired toxic side effects and unwanted isomerization products (Blom et al., 2001). A linear increase was applied (acetonitrile from 20% to 70% in 50 min, 70–100% in 2 min, isocratic for additional 10 min). Fractions were collected every 3 min; the solvents were evaporated by vacuum centrifugation (SPEEDVAC Plus® SC110A, Savant Instruments Inc., USA). The fractions containing the different cyanobacterial compounds were transferred to a 24 well plate, and re-dissolved in 1% MeOH (500 μL). The toxicity of the fractions of the six crude extracts was tested in a 24-h acute toxicity assay performed using instar II-III larvae (Thamnotoxkit F; MicroBioTests Inc.) of the sensitive crustacean *Thamnocephalus platyurus*, which can be easily hatched from cysts (Blom et al., 2003, 2001). About 20–30 crustaceans were transferred to each well; after 24 h, the mortality rate was determined for each fraction. Each fraction represented the extract of an equivalent of 0.05 μM chlorophyll a of the biomass of the six *Planktothrix* strains.

2.3. Isolation of aeruginosin 828A

Fresh biomass (32 g) of *Planktothrix* strain 91/1 was extracted with 50% MeOH. Crude extracts were obtained after centrifugation and were separated by HPLC using a reversed phase column (Hydrosphere C18, YMC, 4.6 × 250 mm, Stagroma, Reinach, CH) under the following conditions: solvent A was UV-treated deionised water (+0.05% TFA), solvent B: HPLC-grade acetonitrile (+0.05% TFA); a linear increase was applied (as described above). Under the conditions applied aeruginosin 828A (**1**) eluted after 16.0 min. TFA was removed before evaporating the solvents to prevent undesirable isomerization products by applying sequentially the combined HPLC fractions on a C18 cartridge (10 g; Mega Bond Elute, Varian, Agilent Technologies, Basel, CH; conditioned with 10% MeOH). The cartridge was flushed with water to remove the TFA, and aeruginosin 828A (**1**) was eluted with 80% MeOH. Subsequently, the aqueous methanol was evaporated (35 mbar, 40 °C) to achieve a colorless, amorphous solid. After purification 533 μg of pure aeruginosin 828A (>99% HPLC) could be achieved.

Aeruginosin 828A (**1**): UV (47% acetonitrile in water with 0.05% TFA λ_{max} 277 nm; ^{1}H and ^{13}C NMR data (DMSO-d_6), see Table 1; HRMS-ESI: calcd. for $C_{36}H_{53}{}^{35}ClN_6O_{12}SNa^+$ $[M+Na]^+$: 851.3023; found: 851.3019.

2.4. Acute toxicity of aeruginosin 828A

The highly purified aeruginosin 828A was tested in a 24 h acute toxicity assay with *Thamnocephalus platyurus* in six concentrations ranging from 0.5 to 100 μM in triplicates. For every concentration, 20–30 animals were used. The nonlinear regression analysis as well as the LC_{50} value was calculated using Graph Pad Prism 5 for Windows.

2.5. Enzyme inhibition assays

The inhibition of trypsin (0.04 U/200 μL; No. 9471 Fluka, Buchs, Switzerland), and thrombin (5 nM; IHTa Innovative Research, Peary, USA) was tested in microtiter plates. Boc-Gln-Ala-Arg-aminomethylcoumarin (50 μM; Bachem AG, Bubendorf, Switzerland) served as substrate for trypsin, and Boc-Phe-Ser-Arg-aminomethylcoumarin (100 μM; Bachem AG, Bubendorf, Switzerland) as substrate for thrombin according to previously established protocols (Blom et al., 2006; Gademann et al., 2010).

Table 1
NMR spectroscopic data for aeruginosin 828A.

Aeruginosin 828A (700 MHz, DMSO-d6, 298 K)					
Residue	Position	^{1}H (*J* in Hz)	^{13}C (from 2D)	HMBC[a]	NOE
Xyl	1eq	4.94 d (3.8)	95.0	Xyl 2,3,5, Choi 6	Choi 6, Xyl 2,4′,7,7′
	2ax	3.28 m	71.8	Xyl 3,5	Xyl 1,4
	3ax	3.57 ddd (2.6, 9.0, 9.1)	71.4	Xyl 2,4,5	Xyl 4,5ax, 3-OH
	4ax	3.93 ddd (5.8, 9.1, 10.6)	74.7	Xyl 3,5	Xyl 1,2,5ax,5eq,3-OH
	5ax	3.36 dd (10.6, 10.7)	59.3	Xyl 1,3,4	Xyl 4,5eq
	5eq	3.67 dd (5.8, 10.7)		Xyl 1,3,4	Xyl 4,5ax
	2-OH	4.41 (7.3)	–	Xyl 1,2	–
	3-OH	4.96 (2.6)	–	Xyl 2,3,5	Xyl 3,4
Choi	1	–	171.1	–	–
	2	4.18 dd (9.4, 8.2)	59.5	Choi 1,3	Aeap NH, Choi 3,3′,3a,7
	3	2.01 ddd (12.6, 7.3, 7.3)	30.5	Choi 3a,7a	Choi 2,3a,3′,4′,6,7′,7a
	3′	1.81 ddd (12.6, 12.6, 9.7)	–	Choi 1,2,3a	Choi 2,3,3a,4′,5′,7
	3a	2.25 m	35.6	Choi 3,4,5,7,7a	Choi 2,3′,4′,7,7a
	4	2.14 m	19.1	Choi 3,3a,5	Choi 3a,4′,5,5′
	4′	1.48 m	–	Choi 5,6,7a	Choi 3a,4,5′,6
	5	1.49 m	24.4	Choi 6,7a	Choi 6,Cleu 2
	5′	1.54 m	–	–	Choi 3a,4,5,6,7′
	6	3.83 m	68.4	–	Choi 5,5′,7,7′, Xyl 5ax
	7	1.58 dd (11.9, 12.9)	28.4	Choi 7a	Cleu 2, Choi 6,7′
	7′	2.25 m	–	Choi 5,7a	Choi 2,6,7′,7a, Cleu 2
	7a	4.32 ddd (11.9, 6.4, 6.4)	54.1	Choi 2,3,3a,7, Cleu 1	Choi 3a,4,4′,7,7′, Cleu 2,5
Pla	1	–	172.5	–	–
	2	4.18 (br)	71.5	–	Pla 3, 3′
	3	2.79 dd (14.0, 7.6)	39.7	Pla 1,2,4,5,9	Pla 2,3′
	3′	2.96 dd (14.0, 3.7)	–	Pla 1,2,4,5,9	Pla 2,3
	4	–	137.9	–	–
	5,9	7.23 m	129.5	Pla 3,5,7,9	Pla 3,3′,7
	6,8	7.26 m	127.7	Pla 4,6,8	Pla 5,7,9
	7	7.18 m	125.8	Pla 5,9	–
	2-OH	–	–	–	–
Cleu	1	–	167.4	–	–
	2	4.93 dd (10.7,8.7)	50.9	Cleu 1,3, Pla 1	Cleu 3,4,5, Choi 7a,7,7′
	3	4.00 dd (10.7, 1.8)	68.6	Cleu 1,2,4,5,5′	Cleu 2,4,5′
	4	1.71 dsept. (6.6, 1.8)	27.3	Cleu 5,5′	Cleu 2,3,5
	5	0.87 3H, d (6.6)	15.3	Cleu 3,4,5′	Cleu 2,3,4, Choi 7′,7a
	5′	0.86 3H, d (6.6)	20.6	Cleu 3,4,5	Cleu 2,3,4, Choi 7′,7a
	NH	7.68 d (8.7)	–	–	Cleu 3
Aeap	1	3.16 dddd (13.0, 6.5, 6.5, 5.7)	36.3	Aeap 2,3, Choi 1	Aeap 2,4,5,6,NH, Choi 2
	1′	3.23 dddd (13.0, 6.5, 6.5, 5.7)	–	Aeap 2,3, Choi 1	Aeap 2,4,5,6,NH, Choi 2
	2	2.25 2H, m	28.1	Aeap 1,3,4,6	Aeap 1,1′,4,5,6,NH
	3	–	136.1	–	–
	4	5.61 t (1.6)	119.0	Aeap 2,3,5,6	Aeap 1,1′,2,5,6,NH
	5	4.07 2H, d (1.6)	53.6	Aeap 3,4	Aeap 1,1′,2,4
	6	4.07 2H, s	54.9	Aeap 3,4	Aeap 1,1′,2,4
	NH	8.00 dd (5.7, 5.7)	–	Aeap 1, Choi 1	Aeap 1,1′,2,4,5,6, Choi 2,3,3′
	8	–	154.9	–	–

[a] HMBC correlations are given from proton(s) stated to the indicated carbon atom.

All fluorescent substrates were dissolved in H_2O (5% DMSO). The reaction solution consisted of 140 μL Tris buffer (50 mM Tris–HCl buffer pH 8.0, 150 mM NaCl, 1 mM $CaCl_2$ and 0.1 mg mL^{-1} BSA), 10 μL enzyme solution, 30 μL toxin solution, and 20 μL fluorescent substrate; samples were measured at 37 °C for 20 min in a Fluorescence plate reader (SpectraMAX, GeminiXS, Molecular Devices Corp., USA) with the excitation wavelength at 380 nm and the emission wavelength at 440 nm.

3. Results and discussion

3.1. Bioassay-guided fractionation

Methanolic extracts of the biomasses of six *Planktothrix* strains (Fig. 1) were fractionated and assayed against the sensitive freshwater crustacean *Thamnocephalus platyurus* for acute toxicity. Overall, more than 35 compounds were present in these fractions, and out of total 72 fractions, 12 were found to be highly toxic. In these fractions, three compounds were assigned to known MCs, seven to unknown (sulfate-containing) cyanopeptolins, and 10 were sulfate and chlorine containing peptides of the size of aeruginosins (Fig. 1).

3.2. Structure elucidation of aeruginosin 828A

In order to elucidate the structure of the unknown toxin, the major toxic oligopeptide of *P. rubescens* strain 91/1 was purified and analyzed by NMR spectroscopy. The putative molecular ions at *m/z* = 851.3019 $[M+Na]^+$ and *m/z* = 853.3003 $[M+Na]^+$ in the HRMS spectrum support a molecular formula of $C_{36}H_{53}{}^{35}ClN_6O_{12}SNa^+$ or of $C_{36}H_{53}{}^{37}ClN_6O_{12}SNa^+$ for the respective Na adduct. In addition, the mass spectrum suggests the presence of a sulfate group by the fragment with *m/z* = 749, as well as the presence of one Cl atom with its characteristic isotope pattern (Fig. S1, Supplementary material). The ^{1}H NMR spectrum of aeruginosin 828A (**1**) (Table 1; Fig. S2 Supplementary material) displays two sets of characteristic

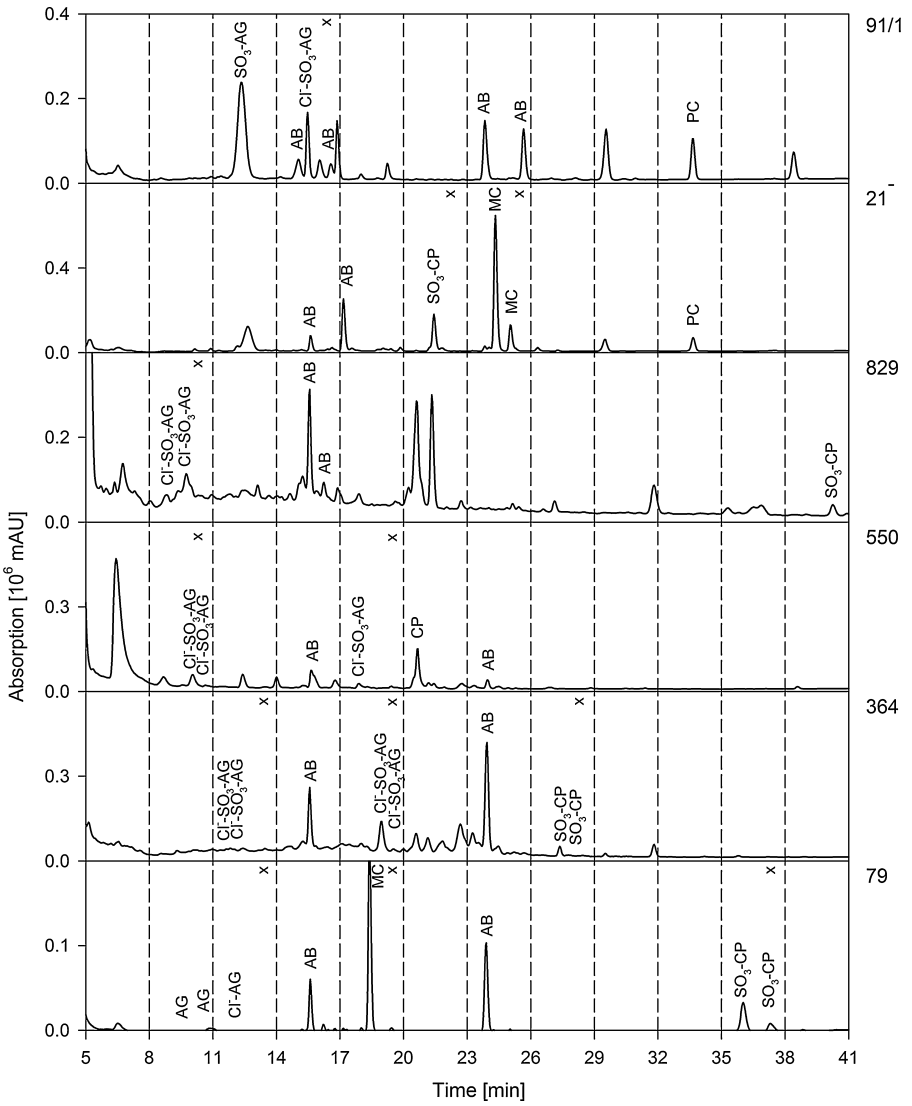


Fig. 1. HPLC-chromatograms (220 nm; 0.05 μM chlorophyll a equivalent) of all six *Planktothrix* strains (AB = Anabaenopeptin, AG = Aeruginosin, Cl^{-}-AG = Cl^{-}-Aeruginosin, Cl^{-}-SO_3-AG = Cl^{-}-SO_3-Aeruginosin, CP = Cyanopeptolin, SO_3-CP = SO_3-Cyanopeptolin, MC = Microcystin, PC = Planktocyclin; *x* = fraction was toxic to *T. platyurus*).

peptide resonances, among others. The constitution of each of the building blocks was assigned using COSY, HSQC, HMBC and NOESY experiments, and the chemical shifts as well as key HMBC and NOE correlations are reported in Table 1. These 2D NMR experiments and comparison to literature data led to the identification of the following building blocks: phenyllactic acid (Pla); chloroleucine (Cleu); 2-carboxy-6-(4′-sulfo-xylosyl)-octahydroindole (Choi) fragment and 3-aminoethyl-1-*N*-amidino-Δ-3-pyrroline residue (Aeap). The position of the Cl atom in Cleu was assigned based on the characteristic chemical shift of the corresponding Cβ atom of the Leu moiety (δ_c = 68.6 ppm). The presence of a carbohydrate residue could be detected due to the low field shifted carbon atoms ($\delta_c \approx 70$ ppm), which indicated the presence of oxygen substituents. The xylose was identified based on the typical proton and hydroxy group substitution pattern. The position of the sulfate group was established by the typical chemical shift of Xyl C-4 (δ_c = 74.7 ppm), by the COSY correlations between H-2 (δ_H = 3.28 ppm) and Xyl OH-2 (δ_H = 4.41 ppm), as well as between Xyl H-3 (δ_H = 3.57 ppm) and Xyl OH-3 (δ_H = 4.96 ppm) (Table 1; Figs. S3 and S4, Supplementary material). The relative configuration of the carbohydrate moiety was assigned on the basis of the NOESY spectrum and *J*-coupling constants. The α-anomer of the sugar moiety was assigned by its small coupling constant (J = 3.8 Hz), which results from the (+)-*sc* arrangement between Xyl H-1 to Xyl H-2. The configuration of the Choi moiety was assigned using the relevant NOE correlations between Choi H-3′ (δ_H = 1.81 ppm) and Choi H-4′ (δ_H = 1.48 ppm), as well as Choi H-4 (δ_H = 2.14 ppm) and Choi H-3a (δ_H = 2.25 ppm), Choi H-3 (δ_H = 2.01, 1.81 ppm) to Choi H-3a (δ_H = 2.25 ppm) and, additionally, to Choi H-6 (δ_H = 3.83 ppm) and Choi H-7′ (δ_H = 2.25 ppm). The configuration at the C-6 stereogenic center of Choi was assigned on the basis of the line width of its signal (total ∼ 16 Hz), which implies that no trans-diaxial relationship of the Choi H-6 to its coupling partners is present. Due to the lack of commercially available, authentic standards for the Cleu and Choi residues, we did not perform hydrolysis and subsequent analysis on chiral stationary phases. The observed NOE between Choi H-3a and Choi H-7a supports the relative *cis*-configuration, which is further substantiated by numerous literature examples of identical configurations. The tentatively assigned relative configuration of aeruginosin 828A (**1**) is further corroborated by comparison with data reported for the structurally similar aeruginosin 205B (Table S2, Supplementary material) (Hanessian et al., 2009). This natural aeruginosin differs only by an agmatine moiety instead of the Aeap residue and shows almost identical ^{1}H and ^{13}C shifts. We therefore assign the relative configuration for the Pla and Cleu building

blocks as shown for compound **1**. Yet, we are aware that its final structure elucidation will require the use of total chemical synthesis.

The assembly of the different fragments was established by HMBC and NOESY experiments (Figs. S5 and S6, Supplementary material). The linkage between central Choi and the Cleu was established by a HMBC correlation between Choi H-7a (δ_H = 4.32 ppm) and Cleu C-1 (δ_c = 167.4 ppm). In a similar fashion, the connection between the Choi and the Aeap residues was determined by the coupling between Aeap H-1 and H-1′ (δ_H = 3.16 ppm and 3.23 ppm) and Choi C-1 (δ_c = 171.1 ppm). The HMBC correlation from Cleu H-2 (δ_H = 4.93 ppm) to Pla C-1 (δ_c = 172.5 ppm) unambiguously demonstrated the connection of these moieties. In addition, the connection of the Xylose unit with the Choi moiety was elucidated by the NOE correlation of the Xyl H-1 (δ_H = 4.94 ppm) to Choi H-6 (δ_H = 3.83 ppm) as well as by the HMBC correlation of the Xyl H-1 (δ_H = 4.94 ppm) to Choi C-6 (δ_c = 68.4 ppm).

3.3. Protease inhibitory activities

Aeruginosin 828A (**1**) belongs to a large group of linear peptides that feature the (hydroxyl)phenyllactic acid (Hpla/Pla) or glyceric acid at the N-terminus (position 1), followed by a variable amino acid in position 2, the Choi moiety in position 3, and an arginine derivative at the C-terminus in position 4 (Welker and von Döhren, 2006). These peptides have been evaluated for high inhibitory activity against trypsin and trypsin-like serine proteases such as thrombin. Aeruginosin 828A (**1**) exhibited strong inhibition toward these proteases: an IC_{50} value of 21.8 nM was measured for thrombin, an IC_{50} value of 112 nM for trypsin (Fig. 1). Thus, aeruginosin 828A (**1**) is one of the strongest inhibitors of trypsin-like enzymes among the aeruginosins, together with chlorodysinosin A, and the aeruginosins 205A and B (Hanessian et al., 2006; Shin et al., 1997; Toyooka et al., 2003). Common to these four aeruginosins is the presence of a Cleu in position 2 that was so far unknown for natural products (Hanessian et al., 2006), and of either a sulfated glyceric acid derivative at the N-terminus or a sulfated Choi subunit. A comparison of the biological activity of other aeruginosins led to the hypothesis that both, chlorine and sulfate moieties are necessary for strong inhibition and toxicity of these peptides (Fig. 2).

It was proposed that the Choi sulfate group would increase the selectivity for trypsin rather than for thrombin (Sandler et al., 1998), and that the sulfate group in the N-terminal position would

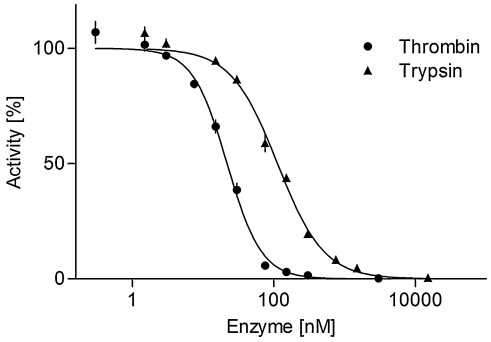


Fig. 2. Trypsin and thrombin inhibition curves of aeruginosin 828A (**1**).

make a significant contribution upon inhibiting thrombin (Carroll et al., 2004). This is supported by the hydrogen bonding network of the dysinosin A–thrombin complex (Carroll et al., 2002). The N-terminated sulfate group of dysinosin A interacted with arginines 208 and 263 of the thrombin with several hydrogen bonds, whereas the 5,6-dihydroxyoctahydroindole group did not appear to have any hydrogen bonding interactions with thrombin. A structure and activity comparison of different aeruginosins revealed that the inhibition values for trypsin of Choi sulfated aeruginosins were lower than the IC_{50} values for thrombin and that the presence of the N-terminal sulfate group considerably decreased the IC_{50} values for thrombin (Table 2, groups 2 and 3). The IC_{50} values for the inhibition of trypsin are typically in the low micromolar range. However, for a strong inhibition of thrombin in the low nanomolar range, the N-terminal sulfate group seems to be essential (Table 2, groups 2 and 3). The inhibition values for aeruginosin 828A seem to be in opposition to this more general trend. However, other factors, such as the arginine derivative in position 4 might have an additional influence on the inhibition potential toward trypsin-like proteases as it was shown for the guanidino group of dysinosin A that strongly bonded to aspartic acid 229 of thrombin (Carroll et al., 2002). Future experiments by using derivatives of aeruginosin 828A might address this conundrum.

The absence of chlorine was thought to be responsible for the comparatively high concentration of aeruginosin 98B that was required for plasmin and thrombin inhibition (Sandler et al., 1998). However, the comparison of different aeruginosins revealed that a chlorine at the N-terminus does not significantly contribute to the inhibition of trypsin-like proteases (Table 2, groups 2 and 4). Only

Table 2
Structure characteristics and enzyme inhibition of aeruginosins (that are characterized by the following structure: Pla/Hpla/Glyceric acid – Amino acid – Choi – Arg derivative).

Group	Aeruginosin example (number of peptides evaluated)		Modifications (Cl, chlorination; Br, bromination; SO_3, sulfation)			Enzyme inhibition (IC_{50})[g]	
			Pla/Hpla/Glyceric acid	Amino acid	Choi	Trypsin [μM]	Thrombin [μM]
1	Chlorodysinosin A[a]	(1)	SO_3	Cleu	–	0.037	0.0057
	Aeruginosin 828A[b]	(3)	–	Cleu	SO_3	0.09–0.112	0.022–1.9
2	Aeruginosin 89A[c]	(2)	Cl, SO_3	Leu	–	0.6–9.2	0.04–0.07
	Aeruginosin 98A[d]	(6)	Cl, Cl_2, Br, Br_2	Ile	SO_3	0.1–18.2	3.6 to >45.5
3	Aeruginosin 102-A[e]	(6)	SO_3	Tyr/Val/Leu	–	0.27–1.5	0.05–0.14
	Aeruginosin 98B[d]	(1)	–	Ile	SO_3	0.9	15.3
4	Aeruginosin GE 686[f]	(5)	Cl, Cl_2, Br, Br_2	Ile/Leu	–	2.2–8.5	12.8 to >45.5

[a] Hanessian et al. (2006).
[b] This study.
[c] Ishida et al. (1999).
[d] Murakami et al. (1995).
[e] Matsuda et al. (1996).
[f] Elkobi-Peer et al. (2012).
[g] IC_{50} values reported in mg mL^{-1} were converted to μM.

the chlorine of Cleu in position 2 might indicate an additional positive effect upon inhibiting proteases such as trypsin and thrombin, as indicated by the lowest IC_{50} values for these aeruginosins (Table 2, group 1).

3.4. Toxicity of aeruginosin 828A

Aeruginosins have been long known for their inhibition potential toward trypsin-like serine-proteases, however, toxicity was not described to date (Ersmark et al., 2008; Nagarajan et al., 2013). Assessment of acute toxicity of aeruginosin 828A (**1**) toward *Thamnocephalus platyurus* revealed a LC_{50} value of 22.4 μM. This value is comparable to those determined for MCs, e.g. a LC_{50} value of 10.8 μM was measured for MC-LR (Blom and Jüttner, 2005). This clearly shows that aeruginosin 828A (**1**) represents another toxin in *Planktothrix rubescens* strain 91/1, a strain that has lost the ability to produce MCs due to a point mutation within the *mcy* gene cluster. Such toxicity might be also unveiled for other aeruginosins. Bioassay-guided fractionations showed strong toxicity of aeruginosins only, if they contain both, chlorine and sulfate (Fig. 1). Aeruginosins bearing only sulfate, or containing neither sulfate nor chlorine did not seem to be toxic to *T. platyurus* in the concentrations tested (e.g. *P. rubescens* strain 79, fraction 2; *P. rubescens* strain 91/1, fraction 3; Fig. 1).

Water-bloom forming cyanobacteria such as *Planktothrix* are producing several bioactive substances, most prominently MCs that are the most abundant toxins in freshwater. Besides the fact that MCs strongly inhibit the protein phosphatases 1 and 2A, the ecological function of MCs is still not clear. However, it is widely agreed that MCs reduce significantly the survival rate of grazers such as crustaceans (Blom et al., 2006; Kurmayer and Jüttner, 1999; Rohrlack et al., 1999). Blooms of cyanobacteria are typically composed of microcystin- and non-microcystin-producing strains that evolved independently (Christiansen et al., 2008). In this study, we investigated the toxicity of aeruginosin 828A that was found in a MC-deficient strain. However, MCs and aeruginosins clearly inhibit a different spectrum of enzymes pointing to a different mode of action. In future, it will be necessary to consider not only the MCs but also compounds such as aeruginosin 828A to forecast the toxicity of cyanobacterial blooms and to unravel their impact on various aquatic organisms.

4. Conclusions

In this note, we have reported the structure elucidation of aeruginosin 828A (**1**) isolated from *Planktothrix rubescens* strain 91/1, which inhibits trypsin and thrombin in the low nanomolar range. Additionally, aeruginosin 828A (**1**) was found to be toxic for the freshwater crustacean *Thamnocephalus platyurus*, and its toxicity was determined to be only slightly lower than MCs that are the prototypical examples of peptide toxins in cyanobacteria. This report thus suggests the presence of alternative toxins in MC-deficient *Planktothrix* strains and encourages further evaluation of the presence and toxicity of modified aeruginosin variants in *Planktothrix* strains.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.hal.2014.07.003.

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CHAPTER 3 - EFFECTS ON THE AQUATIC MODEL ORGANISMS ZEBRAFISH AND DAPHNIA MAGNA

MANUSCRIPT III

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Molecular effects of the cyanobacterial toxin cyanopeptolin (CP1020) occurring in algal blooms: Global transcriptome analysis in zebrafish embryos

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ABSTRACT

Higher water temperatures due to climate change combined with eutrophication of inland waters promote cyanobacterial blooms. Some of the cyanobacteria produce toxins leading to drinking water contamination and fish poisoning on a global scale. Here, we focused on the molecular effects of the cyanobacterial oligopeptide cyanopeptolin CP1020, produced by *Microcystis* and *Planktothrix* strains, by means of whole-genome transcriptomics. Exposure of 72 hpf old zebrafish embryos for 96 h to 100 and 1000 μg/L CP1020 resulted in differential transcriptional alteration of 396 and 490 transcripts (fold change ≥2), respectively, of which 68 gene transcripts were common. These belong to genes related to various important biological and physiological pathways. Most clearly affected were pathways related to DNA damage recognition and repair, circadian rhythm and response to light. Validation by RT-qPCR showed dose-dependent transcriptional alterations of genes belonging to DNA damage and repair and regulation of circadian rhythm. This leads to the hypothesis that CP1020 acts on DNA and has neurotoxic activity. This transcriptome analysis leads to the identification of novel and unknown molecular effects of this cyanobacterial toxin, including neurotoxicity, which may have important consequences for humans consuming contaminated drinking water.

1. Introduction

Cyanobacteria can form dense blooms in many aquatic systems, one of the most bloom triggering factors being the nutrient load. Due to eutrophication, an increased frequency of cyanobacterial blooms may be observed worldwide (Carmichael, 2008). Furthermore, this increase can be related to global warming (Paerl and Paul, 2012; Posch et al., 2012). Thick mats of cyanobacteria deteriorate recreational areas, can block the sunlight from reaching deeper water layers and cause an anoxic environment during decomposition.

Cyanobacterial blooms raise concerns about their ecotoxicological and human health implications due to the formation of bioactive secondary metabolites. Their presence is frequently observed in different cyanobacterial genera such as *Microcystis*, *Anabaena*, and *Planktothrix*. Some of the most toxic natural compounds are found among cyanobacterial toxins, including the highly hepatotoxic microcystins (MC). Microcystins are among the most abundant cyanobacterial toxins, mainly produced by *Microcystis* and *Planktothrix* species (Sivonen and Jones, 1999). During decomposition of toxic and so-called harmful algal bloom, these toxins contaminate waters, causing illness or death of other organisms including humans (Azevedo et al., 2003; Poste et al., 2011). MCs are cyclic heptapeptides that inhibit serine/threonine-specific protein phosphatases 1 and 2A (McKintosh et al., 1990), and that bind to the beta chain of the ATP-synthase unit (Mikhailov et al., 2003). Moreover, MCs lead to endoplasmic reticulum stress (Christen et al., 2013) and, due to their tumor promoting activity, they are considered to be responsible for an increased rate of liver cancer in China (Ueno et al., 1996) and Serbia (Svirčev et al., 2009). For MC-LR, the most abundant of more than 90 different MC congeners, WHO set a provisional guideline in drinking water of 1 μg/L (Burch, 2008).

Due to its high toxicity, research has mainly focused on MC, whereas other cyanobacterial toxins have received only little

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attention. However, there is a great diversity of cyanobacterial secondary metabolites, including oligopeptides, but their potential adverse effects to organisms remain largely unknown. Cyanopeptolins (CP) are peptides widely distributed amongst cyanobacteria, and mainly known for their inhibition of serine proteases like chymotrypsin or trypsin (Blom et al., 2006; Bister et al., 2004). CP1020 was isolated from *Microcystis* and its acute toxicity (LC_{50} = 8.8 μM) to the crustacean *Thamnocephalus platyurus* was comparable to that of microcystins (Blom and Jüttner, 2005). Moreover, CP1020 showed very potent inhibitory activity to crustacean and mammalian serine proteases (Gademann et al., 2010). Similar to MC, CP1020 is assumed to enter fish cells by active transport processes. However, so far nothing is known about its uptake mechanism, molecular effects and modes of action to fish and mammals.

In general, the biological functions and toxic action of cyanobacterial oligopeptides are not well understood. Effects of natural and anthropogenic compounds on the gene expression level can occur even before physiological consequences can be observed (Christen et al., 2011; Oggier et al., 2010; Yang et al., 2007; Zucchi et al., 2011). Therefore, investigations of transcriptional alterations can provide insights into molecular mechanisms underlying a toxic response. In addition, transcriptomal effects in zebrafish may be a surrogate for effects in mammals. Using global transcription analysis (transcriptomics), mRNA expression pattern of thousands of genes can be analyzed simultaneously, allowing a detailed comparison between organisms exposed to a toxin and control organisms (Fent and Sumpter, 2011). Recently, molecular effects of MC-LR on zebrafish embryos have been analyzed by transcriptome analysis (Rogers et al., 2011), the only cyanobacterial toxin investigated by this advanced method in fish so far. The aim of the present work was to evaluate the molecular effects and mode of actions of the novel cyanobacterial toxin CP1020 in zebrafish eleuthero-embryos by means of microarrays. The obtained data shed new lights on the potential ecotoxicological and human health risks originating from toxins from cyanobacterial blooms, which are of increasing concern due to eutrophication and global warming.

2. Materials and methods

2.1. Culture conditions of cyanobacteria

Microcystis aeruginosa strain UV006 was cultured in 300 mL Erlenmeyer flasks at 20 °C under constant light conditions at an irradiation of 6 μmol/m^2 s from fluorescent tubes (Osram 930; Lumilux Delux; Warm White 3000 K) in 120 mL mineral medium (Jüttner et al., 1983).

2.2. Separation and mass spectrometrical analysis of the oligopeptides

Frozen biomass was extracted twice with 60% MeOH (10 mL per g wet weight) for 2 h in the dark. After centrifugation (25,700 × *g* for 15 min) the supernatant (crude extract) was separated by HPLC equipped with a photodiode array detector using a reversed phase column (Hydrosphere C18, YMC, 4.6 mm × 250 mm, Stagroma, Reinach, CH; ODS-A, 4.6 mm × 250 mm, Stagroma, Reinach, CH) under the following conditions: solvent A was UV-treated deionised water (+0.05% trifluoroacetic acid; TFA), solvent B: HPLC-grade acetonitrile (+0.05% TFA). A linear increase in three steps was applied (solvent B: from 30% to 35% in 10 min, from 35% to 70% in 30 min, 70 to 100% in 2 min, isocratic for additional 10 min). Mass spectra were recorded on a combined LC–MS (LCQ Duo mass spectrometer, Finningan Thermoquest, USA) equipped with an electrospray ionization source (ESI-MS). Cyanopeptolin 1020 was collected by HPLC and purified to eliminate TFA as it may lead to undesirable isomerization products of some oligopeptides by using C18 cartridges (1 g, 60 mL, Mega Bond Elute, Varian, Agilent Technologies, Basel, CH) and MeOH (ROTISOLV ≥99.95%, LC-MS-Grade, Roth, CH). Chromatograms of absorption and masses of the detected CP1020 are shown in the supplementary data (Figure S1). Purity was found >98% by absorption.

2.3. CP1020 preparation

A 10 mM stock solution was prepared from the lyophilized CP1020 in dimethyl sulfoxide (DMSO; from Sigma–Aldrich, Fluka AG, Buchs, Switzerland) and further diluted to two stock solutions of 1 g/L and 10 g/L CP1020. The latter was then diluted with reconstituted fish water to obtain final nominal concentrations of 100 μg/L and 1000 μg/L CP1020 (0.01% DMSO).

2.4. Eleuthero embryos exposure

Fertilized eggs were obtained from Harlan Laboratories Ltd. (Itingen, Switzerland). After quality control under the stereomicroscope (Zeiss, D4), early embryos were transferred to 150 mL glass beakers (80 embryos per beaker, 16 beakers in total) containing 100 mL of freshly prepared reconstituted fish water (deionized water with ions added: 61.6 mg/L $MgSO_4 \times 7\ H_2O$; 147 mg/L $CaCl_2 \times 2H_2O$; 32, 4 mg/L $NaHCO_3$ and 2.9 mg/L KCl and a conductivity of 470–480 μS/cm). Beakers were covered with Petri dishes and held in an incubator with temperature set to 27 ± 1 °C and a photoperiod of 16:8 h light/dark for development of zebrafish embryos.

Similarly to Rogers et al., 2011, static exposure started at 72 h post fertilization (hpf). A total of 60 hatched eleuthero-embryos out of the 80 embryos from each beaker were transferred into a new autoclaved beaker containing 100 mL of freshly prepared media with the appropriate concentrations of CP1020. Exposure to CP1020 was performed in four concentration groups, water control, DMSO solvent control (0.01% DMSO), 100 and 1000 μg/L CP1020. For each concentration group, 4 replicates (independent biological replicates) at each nominal concentration of 100 and 1000 μg/L and 4 replicates of water control and solvent control (0.01% DMSO) were included.

Limited quantity of purified Cyanopeptolin for eleuthero-embryo exposure reduced the overall amount of exposure water collected for chemical analysis and the total number of water exchanges performed during the exposure period. In fact, exposure water (100 mL) was taken at the beginning (0 h) from freshly prepared dilutions and after 4 days (96 h) of exposure, directly from each of the four replicates. Samples were stored at −20 °C until further analysis by HPLC.

Every 24 h, normal development and viability was controlled under the stereo microscope. Zebrafish eleuthero-embryos were sacrificed after 96 h (168 hpf) for RNA extraction and subsequent microarray analysis.

2.5. Total RNA extraction, microarray hybridization, and sample selection

Eleuthero-embryos from each replicate (n = 4) were randomly separated into two groups of 30 individuals, pooled in RNA-Later and stored at −80 °C. Total RNA was extracted from zebrafish pools (n = 30) using the RNeasy Mini Kit (Qiagen, Basel, Switzerland). RNA concentrations and RNA purity were measured spectrophotometrically using a NanoDrop ND-1000 UV-VIS spectrophotometer, and RNA integrity was controlled using the Biorad Experion automated electrophoresis system, and an Agilent 2100 Bioanalyzer (Agilent Technologies, Basel, Switzerland). Only samples with a 260/280 nm ratio between 1.8 and 2.1, and an RNA integrity number (RIN) > 8

were used for the hybridization. RNA processing and hybridization for transcriptome analysis was performed by the Functional Genomics Centre (FGCZ), ETHZ and University of Zürich. Global transcriptome analysis was performed as previously described (Oggier et al., 2010; Zucchi et al., 2011).

For transcriptomic analysis, two CP1020 concentrations and the solvent control were used. Transcriptional changes induced by CP1020 were determined by comparison of CP1020-treated embryos to those of the solvent control treated with the identical amount of solvent (0.01% DMSO). A total of 12 arrays (Agilent 4 × 44 K Zebrafish microarray, each array contains barely 43803 probes), were used, array was used for each replicate and three independent arrays were used for each treatment. 600 ng of total RNA were reverse-transcribed into double strand cDNA in the presence of RNA poly-A controls with the Agilent One Color RNA Spike-In Kit. Cy3 labeling and hybridization were performed by the Functional Genomics Centre (FGCZ), ETH and University of Zürich, according to the manufacturer's manual.

2.6. RT-qPCR analysis

Validation of microarray results was performed by real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis of selected target genes including nuclear receptor subfamily 1 (*nr1d1*), period homolog 1a (*per1a*), cryptochrome 5 (*cry5*), prostaglandin D2 synthase (*ptgds*), vitellogenin type 2 (*vtgII*), estrogen receptor alpha (*esr1*), estrogen receptor beta 1 (*esr2b*), aryl hydrocarbon receptor nuclear translocator 2 (*arnt2*), *Danio rerio* ATP-binding cassette, sub-family G, member 2a (*abcg2a*), and sub-family C (CFTR/MRP), member 2 (*abcc2*) (Table S1). The gene encoding ribosomal protein RPL was used as internal standard (housekeeping gene). Gene specific primers were obtained from published sequences or designed using NCBI Primer Blast and listed in Table S1.

First, 1 μg RNA was reverse-transcribed by Moloney murine leukemia virus reverse transcriptase (Promega Biosciences, Inc., Wallisellen, Switzerland) in the presence of random hexamers (Roche) and deoxynucleoside triphosphate. Subsequently the reaction mixture was incubated for 5 min at 70 °C and then for 1 h at 37 °C, and at the end for 5 min to 95 °C to stop the reaction. The mRNA quantity was then determined using SYBR green (SYBR green PCR master mix; Roche) in a Biorad CFX 96 Real Time System. Amplification conditions were 95 °C for 5 min, 40 cycles of 95 °C for 30 s, and 57–60 °C at primer specific annealing temperatures (*cry5* and *nr1d1* 62.5 °C; *per1a*, *esr2b*, *arnt2*, *abcc2* and *vtgII* 60.7 °C; *ptgds* and *esr1* 58.6 °C) for 60 s. A melting curve post run (65–95 °C) was performed to confirm the specificity of the chosen primers as well as the absence of primer dimers. In addition, correct PCR product sizes were checked by an agarose gel. Each reaction was run at least in duplicate.

Efficiency of the PCR reactions was determined by generating a standard curve. Ct values resulting from a reaction mixture with template diluted 1:10 in four steps were plotted against the log of the starting quantity. Expression levels of selected genes were calculated using the $2^{\Delta\Delta Ct}$ method. All gene expression data are reported as log 2 transformed.

2.7. Data analysis and statistics

The raw microarray data obtained from the FGCZ were processed according to Oggier et al. (2010) and Zucchi et al. (2011), using GeneSpring GX 11.5 software (Agilent Technologies). Here we show the data only for strong alteration with minimally two-fold difference compared to the solvent control (FC ≥ 2) (supplementary information). In a first step, the Agilent Feature extraction software output was filtered on the basis of feature saturation, non-uniformity, pixel population consistency, and signal strength relative to the background level (Agilent Feature Extraction Manual). Only positively marked entities, in which at least 50% of the values for two out of three conditions, were accepted for further evaluation. All data were quantile normalized. In a second step, several quality control steps (correlation plots and correlation coefficients) using the quality control tool of GeneSpring were performed to ensure that the data were of good quality. In addition, a quality report was provided by the FGCZ, sample clustering is shown in Fig. 2.

Differentially expressed genes (here and elsewhere where the term gene expression is used, it is used as a synonym for gene transcription) from the microarray were determined using a Benjamini–Hochberg multiple correction-ANOVA test ($p < 0.05$ and the fold change (FC absolute ≥2). To determine gene ontology (GO) categories of differentially expressed genes, the GO analysis tool in GeneGo (GeneGo, San Diego, CA, Version 6.3, http://www.genego.com) was used. Enrichment was examined in all three major GO categories (e.g., biological process, cellular component, molecular function), but only biological process results are reported here, as they were the most relevant category for the purposes of this study. Only those categories where $p < 0.05$ are considered differentially altered. MetaCoreTM (GeneGo, San Diego, CA, Version 6.3) from GeneGo Inc. (http://www.genego.com) was used to identify and to visualize the involvement of the differentially expressed genes in specific pathways (FDR < 0.05). Data from qRT-PCR were illustrated graphically with GraphPad_Prism 5 (GraphPad Software, San Diego, CA, USA). Differences between treatments were assessed by one-way ANOVA followed by a Tukey's test (Bartlett's test $p > 0.05$) to compare means of treatments with (solvent-) controls. Results are given as means ± standard error of means. All transcriptomics data are reported as log 2 transformed here.

Table 1
Nominal and mean values of determined CP1020 concentrations in fish media at the beginning (0 h) and end (96 h) of the experiment.

Nominal [μg/L]	Measured [μg/L]	
	0 h	96 h
DMSO control	0	0
100	128.1 ± 7.8	90.1 ± 33.0
1000	1185 ± 13.8	856 ± 57.3

3. Results

3.1. CP1020 concentrations and gross toxicological parameters

The concentrations of CP1020 in exposure waters were close to nominal. At the beginning 128 μg/L and 1185 μg/L CP1020, respectively, were determined in the low and the high dose groups (Table 1). After 96 h the CP1020 concentration decreased to approximately 70% to average concentrations of 90 μg/L and 856 μg/L, respectively. As average concentrations during the 24 h static-renewal exposure were close to nominal, results are presented here as nominal concentrations. No CP1020 was detected in control groups. No mortality or abnormal behavior of zebrafish embryos was recorded during exposure to these concentrations.

3.2. Differential gene expression in CP1020 exposed zebrafish eleuthero-embryos

Exposure to the low and high CP1020 concentrations resulted in differential expression of 390 and 490 genes (fold change ≥ 2, $p \leq 0.05$), respectively as illustrated in the Venn Diagram (Fig. 1)

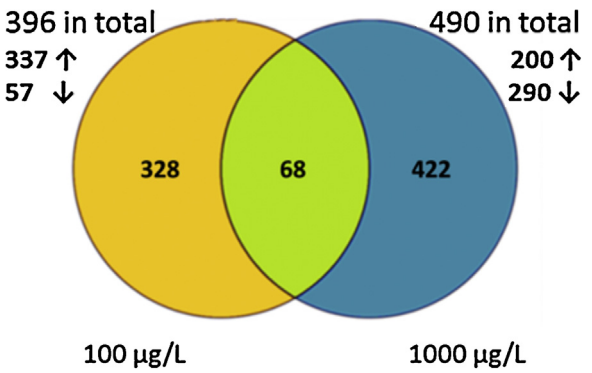

Fig. 1. Venn diagram showing the number of genes that are differentially expressed (fold change≥ 2, $p \leq 0.05$) in the respective treatment group relative to DMSO (0.01%) control. The number of up-regulated and down-regulated transcripts is also shown. The overlapping region represents the number of genes (68) that are altered in common at both CP1020 concentrations.

and listed in the Supplementary Material (Table S2, Table S3, Table S4).

The total set of raw data has been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE50139 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE50139).

Sample clustering analysis (Fig. 2; performed by the Functional Genomic Centre Zurich) clearly revealed partitions among control and treated samples.

Since all hybridizations met the quality requirements, no data were excluded from further analysis. Cluster analysis is given in Fig. 2. Exposure to 100 µg/L CP1020 resulted in 396 significantly (≥2 fold change absolute, $p \leq 0.05$) altered transcripts (328 transcripts unique to this concentration), and exposure to 1000 µg/L in 490 altered transcripts (422 unique to this concentration). A total of 68 genes differentially regulated were common to both treatment groups (Table S2), with 20 (29.4%) up-regulated and 48 (69.1%) down-regulated. One gene, *glrx5* (glutaredoxin 5 homolog 1a), was complementarily regulated in the concentration groups. At 100 µg/L the majority of genes were up-regulated (82.1%), while the majority of genes were down-regulated at 1000 µg/L (59.2%).

Pathway analysis of differentially expressed genes was performed by MetaCore TM software. At both CP1020 concentrations, the top 10 scored pathway maps (revealed by comparison workflow analysis) are listed in Table 2a. Altered transcripts belong to genes and pathways involved in DNA damage and repair, p53 signaling and the neurophysiological process circadian rhythm among others

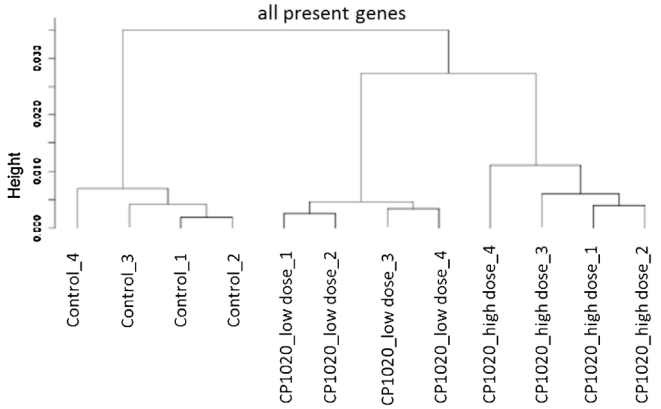

Fig. 2. Cluster analysis of all present genes in each replicate ($n = 4$) from the different exposure groups (control: solvent control (0.01% DMSO); CP1020 low dose: 100 µg/L CP1020; CP1020 high dose: 1000 µg/L CP1020).

Table 2
10 top scored pathways (a), and 10 top GO processes (b) differentially expressed in common at both CP1020 concentrations as represented in MetaCore (FDR< 0.05).

(a)

Top pathways	*p* value
DNA damage_Brca1 as a transcription regulator	1.055e−02
DNA damage_Role of Brca1 and Brca2 in DNA repair	1.055e−02
DNA damage_Nucleotide excision repair	1.266e−02
Transcription_P53 signaling pathway	1.371e−02
Neurophysiological process_Circadian rhythm	1.650e−02
Heme metabolism	3.489e−02
Putative pathways for stimulation of fat cell differentiation by Bisphenol A	1.000e+00
Development_EGFR signaling pathway	1.000e+00
Chemotaxis_CCL2-induced chemotaxis	1.000e+00
Immune response_Regulation of T cell function by CTLA-4	1.000e+00

(b)

Top GO processes	*p* value
Nucleotide-excision repair, DNA damage recognition	9.613e−04
Proline catabolic process to glutamate	1.442e−03
Proline catabolic process	1.922e−03
Negative regulation of toll-like receptor 4 signaling pathway	2.402e−03
Glutamate biosynthetic process	2.881e−03
Porphyrin-containing compound catabolic process	3.361e−03
Tetrapyrrole catabolic process	3.361e−03
Heme catabolic process	3.361e−03
Positive regulation of cholesterol homeostasis	3.361e−03
Pigment catabolic process	3.361e−03

(including heme metabolism). GO processes of genes that respond to CP1020 treatment were also analyzed by MetaCore software and the top ten processes are listed in Table 2b. Strongly regulated are genes belonging to the processes nucleotide-excision repair and DNA damage recognition, catabolic and biosynthetic processes. The lists of altered transcripts unique to 100 and 1000 µg/L, respectively, are given in Tables S3 and S4.

3.3. Validation of microarray data by quantitative real time PCR of selected genes

Based on MetaCore analysis, genes belonging to the top scored pathways and some additional genes were selected for validation of the array data. Additionally, concentration-response relationships of selected genes are evaluated. The obtained microarray and quantitative RT-qPCR data are depicted in Fig. 3 and Figure S2. Genes encoding nuclear receptor subfamily 1, group D member 1 (*nr1d1*), and the period homolog 1a (*per1a*), both involved in circadian rhythm (Vatine et al., 2011), are significantly down-regulated in both concentration groups. This is reflected in the microarray data, and confirmed by RT-qPCR. Cryptochrome 5 (the product of *cry5* gene) is related to circadian rhythm (Cashmore et al., 1999), but functions in DNA damage repair (Hirayama et al., 2009). Down-regulation of *cry5* is demonstrated both by means of microarray and RT-qPCR analysis, showing a good correlation between the two methods. Furthermore, prostaglandin D2 syntethase D2 gene (*ptgds*) is down-regulated, as shown by microarrays and validated by RT-qPCR. Prostaglandin D2 is known to mediate sleep, body temperature and hormone release (Mong et al., 2011).

The microarray data also show transcriptional alterations of estrogen receptor genes *esr1* and *esr2b*, which were also analyzed by RT-qPCR (Figure S2). In addition, two genes for ABC-transporters, *abcc2* and *abcg2a*, and *arnt2* are analyzed by RT-qPCR (Fig. 3, Figure S2), but the alteration found by microarray lack confirmation for *abcc2 and arnt2* and *vtg2* could not be amplified. Thus, alteration of these transcripts of genes involved in endocrine signaling (*esr1*,

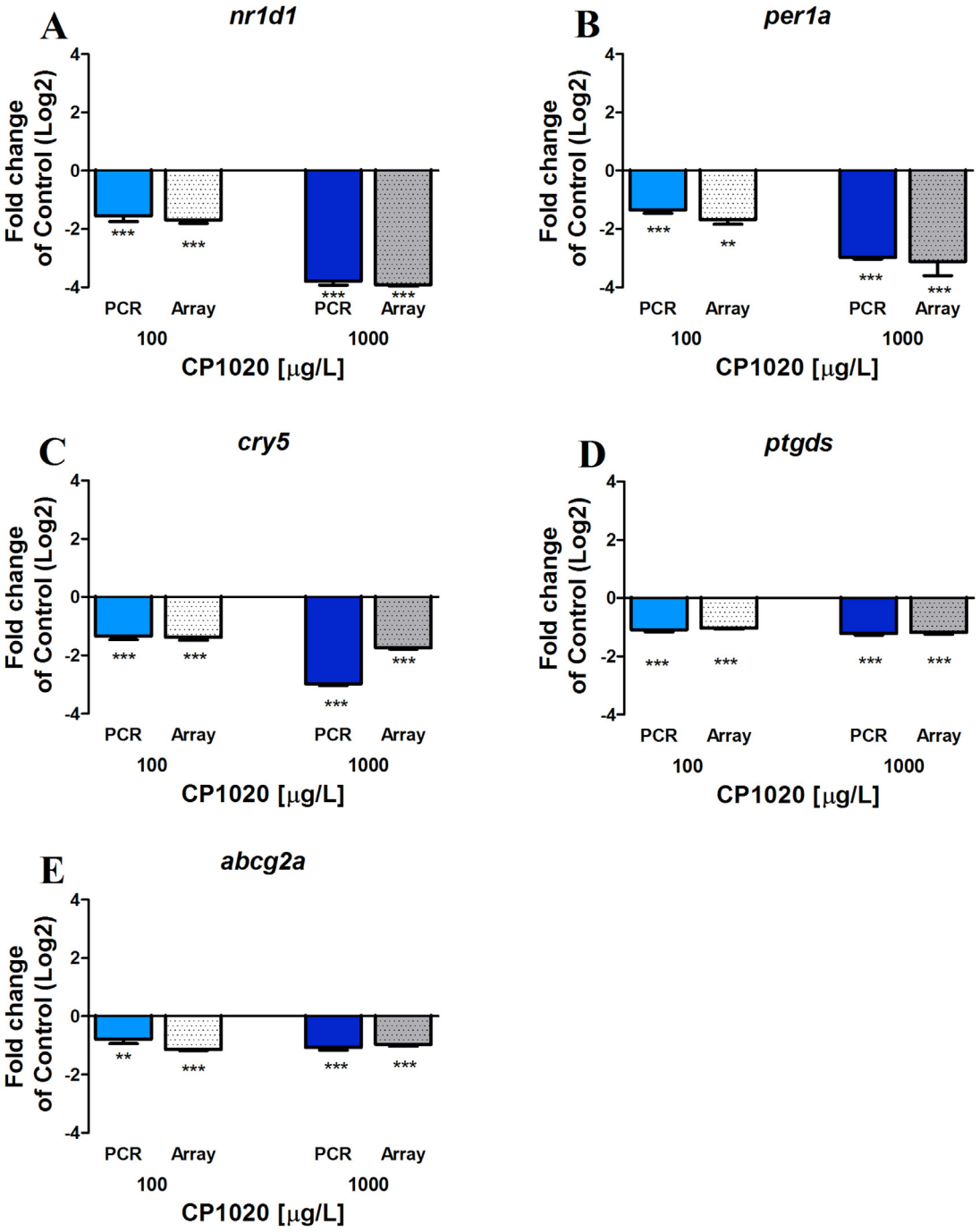

Fig. 3. Relative transcriptional expression (shown as fold change log 2) analyzed by microarray and RT-qPCR of *nr1d1* (A), *per1a* (B), *cry5* (C), *ptgds* (D), *abcg2a* (E) *abcc2* (F), of esr2b (G), esr1 (H) and *arnt2* (I) in zebrafish eleuthero-embryos exposed to 100 µg/L and 1000 µg/L of CP1020, compared to embryos exposed to the solvent control (0.01%DMSO). Results are given as mean ± standard deviation ($n = 4$ replicates per treatment). Significant alterations compared to solvent control are indicated by asterisks ($^{*}p < 0.05$; $^{**}p < 0.001$; $^{***}p < 0.0001$).

esr2b, *vtg2*), and the ABC-transporter gene *abcc2* alteration, as well as *arnt2* alteration seem to be of minor importance.

4. Discussion

This study demonstrates the transcriptional interference of CP1020 with genes involved in many biological and physiological processes in zebrafish. Global transcriptome analysis revealed hitherto unknown mode of actions of this cyanopeptolin. Among many others, the most affected pathways were DNA damage recognition and repair, circadian rhythm, response to light and heme metabolism (provided that the transcriptional change is reflected in the change in gene product activity). Results achieved in this study give first insights into the possible modes of action of CP1020. Forthcoming investigations are needed to further analyze effects on the physiological level based on these transcriptomics data. Exposure to two different CP1020 concentrations resulted in transcriptional alteration of many genes. 68 transcripts, were altered in both concentrations and except for one, they were all regulated in the same direction in the two concentrations, meaning each gene was either up-regulated in both concentrations, or

down-regulated in both concentrations. However, a higher number of genes was altered only in one of the two concentrations. The two top-scored altered pathways, by analyzing transcripts that are altered in both concentration groups, were DNA damage pathways involving the transcription factors and tumor suppressor genes *Brca1* and *Brca2*, which, however, are lacking in fish. Metacore analysis is based on the human genome, and therefore data from zebrafish are translated to human homologs or orthologs by this software. Pathway analysis is therefore related to humans, which indicates some restrictions to data interpretation for zebrafish (Fent and Sumpter, 2011).

Nevertheless, transcripts of important genes involved in DNA damage recognition and repair were differentially expressed at both CP1020 concentrations. The *Xeroderma pigmentosum* complementation group C gene (*xpc*), and the damage-specific DNA binding protein 2 gene (*DDB2*) were down-regulated (Table S2). Both genes are responsible for initiation of nucleotide excision repair (NER), a system that eliminates a wide variety of helix-distorting DNA lesions (Matsuda et al., 2005; Araki et al., 2001). XPC is a key factor in NER and together with *DDB-2* recruiting machinery to eliminate DNA damage (Ray et al., 2013). Defective XPC function results in a cancer prone phenotype. A crucial role in preventing cancer is known for p53 and its activity seems to be affected by CP1020 treatment. P300, activating p53 by covalent modification, was transcriptionally up-regulated at 1000 μg/L (Table S4), while dual specificity phosphatase MKP1 (*dusp-1*), which plays a role in activation of p53 by inhibition of a p53 activating factor, that was significantly down-regulated at 1000 μg/L CP1020 (Table S4). The strong down regulation of *cry5*, the 6–4 photolyase in zebrafish at both CP1020 concentrations is further evidence for affected DNA damage repair by CP1020 (Fig. 3). Photolyases repair DNA adducts induced by UV-light (Sancar, 2003).

The circadian rhythm is regulated by a complex interaction of transcription-translation and posttranslational feedback loops. They consist of core feedback loop genes (Clock and Bmal heterodimers that regulate transcription of Period *per*), cryptochrome (*cry*) genes, and a stabilizing loop (Rev-ERBalpha (*nr1d1*) and Rora regulate expression of the Clock and Bmal genes) (Vatine et al., 2011). In CP1020 exposed zebrafish eleuthero-embryos *nr1d1* was strongly down-regulated at both concentrations (Fig. 3), while *bmal1b* was slightly up-regulated (FC (log 2) 0.8) at 1000 μg/L (data not shown). Furthermore, the transcriptional repressor genes *per1a/per1b*, *nr1d2a* and *nr1d2b* were significantly down-regulated at the high dose (Table S4). Transcripts of *per2a*, also acting as transcriptional repressor, were slightly down-regulated (0.8-fold (log 2)) in both concentration groups. In contrast to these down-regulated transcripts involved in the circadian rhythm, most of the transcripts of opsin genes, *rho*, *opn1mw2*, *opn1sw2*, *opnsw1*, *tmtospa* were significantly or slightly up-regulated at 100 μg/L (Tables S2, S3). Their expression is also regulated by the circadian rhythm (Li et al., 2005) and some genes involved in the regulation of the circadian rhythm are inducible by light, like the D box-binding factor *TEF*, which directs light-induced clock gene expression (Gavriouchkina et al., 2010). Expression of *tef* was strongly down-regulated at 1000 μg/L (Table S4), and slightly (FC 0.6 (log 2)) at 100 μg/L, suggesting that deregulation of circadian rhythm could be influenced by response to light.

Interestingly, in addition to *tef*, other light-inducible genes (cry5, cry-*DASH*, *per2*, si:ch211-195b13.1, zgc:56136, zgc:153154, serum/glucocorticoid regulated kinase 1-like, *xpc*, *ptgds* among others) were also significantly down-regulated by CP1020 (Tables S2, S3, S4). The comparison of our data with the light responsive transcriptome of zebrafish (Weger et al., 2011) suggests that CP1020 negatively influences the reaction onto light stimulus. Furthermore, the top-scored GO process by analysis of transcripts that are only altered in the low dose group was the neurophysiological process "visual perception" (data not shown). Transcriptional processes within the retina involved in visual perceptions were shown to be regulated by *nr1d1* (Mollema et al., 2011) and *nr1d1* was strongly down-regulated in our study. However, in zebrafish the central photoperceptive organ is the pineal gland. The circadian rhythm controls a variety of cellular and physiological processes. Therefore, it seems likely that deregulation of the internal clock has further influence on additional pathways and processes, including hormonal pathways. Prostaglandin D2 is known to mediate sleep, body temperature and hormone release (Mong et al., 2011) and prostaglandin D2 syntethase D2 (*ptgds*) is transcriptionally down-regulated, as shown by microarrays and validated by RT-qPCR. Furthermore, the top scored pathway at 1000 μg/L CP1020 was the Gonadotropin releasing hormone (GnRH) signaling and GnRH is a key hormone related to reproduction in vertebrates. Moreover, significant up-regulation of the estrogen receptor *esr1* at 100 μg/L, down-regulation of *esr2b* and vitellogenin (*vtg2*) up-regulation at 1000 μg/L were shown by microarray data. However this could only partly verified by RT-qPCR (for *esr1*).

The ABC transporter *abcg2a* was altered in both concentrations (Fig. 3). *Abcg2a* is important in heme transport (Desuzinges-Mandon et al., 2011), and influence on heme metabolism was also shown by Metacore pathway analysis. Alteration of another ABC transporter gene, *abcc2*, also shown by microarray data, could not be confirmed by RT-qPCR, and thus seems to be of minor importance.

Exposure of zebrafish to CP1020 affected transcriptional expression of genes belonging to many different pathways. However, it should be noted that only a few transcripts of the affected pathways were altered, and only a relatively small number of transcripts (68 out of 484) showed similar alterations in the low and high dose group. Pathway analyses showed distinct differences in the low and high dose groups.

The global transcription profile clearly differed from that of MC-LR (Rogers et al., 2011) and only a few transcripts altered in common with *Microcystis* treatment (Rogers et al., 2011). Of the nuclear receptor gene family *nr1d2b*, was significantly down-regulated at 1000 μg/L CP1020 (Table S4), as well as by *Microcystis* extracts (Rogers et al., 2011), whereas *nr1d1* was strongly down-regulated at both CP1020 concentrations (Fig. 3), but not by *Microcystis* treatment. Transcripts altered by both *Microcystis* and CP1020 treatment are transcripts of opsin genes, of krueppel like factor (however, another isoform, and only in the high CP1020 dose group) and the thyrotroph embryonic factor *tef* (both function in cell signaling and development) and vitellogenin *vtg*. However, CP1020 induced a different *vtg* isoform, the *vtg II* transcript at 1000 μg/L CP1020 (Table S4), and induction was only six times which is rather low compared to an induction of more than 100 times found in *Microcystis* treatment (Rogers et al., 2011). Furthermore induction by CP1020 could not be confirmed by RT-qPCR. Taken together, the transcriptional profiles of MC-LR and CP1020 are distinct, and only a few of the transcriptional alterations induced by *Microcystis* extracts (Rogers et al., 2011) are also altered by CP1020. The effects of CP1020 found in our study occur at two concentrations, which are assumed to be rather high, but actual concentrations of CP1020 in surface or drinking water are currently unknown. Field sampling would give some indication for the effective environmental concentration. However, cyanopeptolins are widely distributed, not only produced by *Microcystis* species, and there is a high structural variability among them.

In conclusion, we demonstrate that the novel cyanobacterial toxin CP1020 has important transcriptional effects in zebrafish eleuthero-embryos altering a large number of transcripts. Global transcriptome analysis revealed molecular effects and potential modes of action of this toxin, which are distinct from those of MC-

LR. However, the transcriptional response is complex and involves many different, albeit key, biological and physiological processes. The most prominently affected pathways were DNA damage recognition and repair, circadian rhythm, response to light, and to some extent metabolic activities. All of them imply important ecological consequences including neurotoxicity to fish feeding on cyanopeptolin-producing cyanobacteria and human health consequences when drinking contaminated water. Further investigations should focus in detail on the human health and ecological implications of cyanopeptolins. In particular, further investigations should demonstrate as to what extent the hypothetical modes of action on the transcriptional level translate to physiological effects in fish feeding on cyanopeptolin-containing cyanobacteria.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aquatox.2014.01.018.

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Effects of aeruginosin 828A in comparison to microcystin-LR on life-history parameters and selected gene transcripts in *Daphnia magna*

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Abstract

Cyanobacterial mass development poses a toxicological risk to aquatic ecosystems, as these blooms may contain a wide variety of cyanobacterial secondary metabolites. Yet, knowledge of the effects of such cyanobacterial toxins on key freshwater organisms is still limited. We investigated the effects of selected representatives from three cyanobacterial toxin classes on different developmental stages of *Daphnia magna* by evaluation of life-history-parameters and transcription analyses including vitellogenin (*vtg*) and glutathione-S-transferase (*gst*). Chronic exposure of daphnids for 21 d to aeruginosin 828A showed a delay of age at maturation accompanied by an increase in body length and reduced reproduction success. Additionally, aeruginosin 828A caused moulting disruption at concentrations of 100 and 1000 µg/L. Mortality was highest during early developmental stages. Exposure to 1000 µg/L microcystin-LR and aeruginosin 828A caused a strong down-regulation of *vtg* in juvenile daphnids but not in mature animals. Our results suggest that *D. magna* is more susceptible to the studied cyanobacterial toxins during their early developmental stages.

1. Introduction

The anthropogenic impact on freshwaters has strongly favoured cyanobacterial growth during the past century. Moreover, climate change, rising levels of CO_2 and increasing nitrogen in surface waters will specifically promote cyanobacterial growth (O'Neil et al., 2012; Paerl and Huisman, 2008; Posch et al., 2012). High cyanobacterial abundances have a strong influence on herbivorous zooplankton such as *Daphnia*, a keystone organism in freshwaters. Cyanobacteria are of very poor quality as a food source due to the lack of sterols and long-chained polyunsaturated fatty acids (Gulati and DeMott, 1997; Müller-Navarra et al., 2000; von Elert et al., 2003). Additionally, they intracellularly store a range of secondary metabolites that may have detrimental effects on zooplankton upon grazing (Kurmayer and Jüttner, 1999; Rohrlack et al., 2005).

Among these metabolites, microcystins (MCs) have been mainly in the focus of research. MCs inhibit the protein phosphatases 1 and 2a, thus inducing hyperphosphorylation followed by serious cell damage (Eriksson et al., 1990; Ohta et al., 1992). The toxicological effects of MCs are not fully understood. A recent study demonstrated that MC-LR induced endoplasmatic reticulum stress and associated unfolded protein response as well as oxidative stress (Christen et al., 2013). For MCs, several biological functions were suggested, including involvement in photosynthesis, metal chelator activity, predator defence, a role as infochemicals and regulation of gene expression (Blom et al., 2001; Kurmayer and Jüttner, 1999; Schatz et al., 2007). Even allelopathic functions were attributed to MCs, giving their owners distinct advantages in terms of resource competition (Singh et al., 2005). However, strains without the ability to produce MCs are quite common in aquatic ecosystems (Kardinaal et al., 2007; Ostermaier et al., 2013). Many studies still regard MCs as the primary defence mechanisms of cyanobacteria against grazers. However, studies with MC-deficient strains showed similar poor growth of daphnids that might be explained by the presence of protein inhibitors (Kuster and Von Elert, 2013; Lürling, 2003; Semyalo et al., 2009) suggesting that not only MCs may acts as the main defence mechanisms. Aeruginosin 828A (AG 828A), isolated from a MC-deficient *Planktothrix* strain, has been found to be toxic to the freshwater crustacean *Thamnocephalus platyurus*, at a level comparable with the toxicity of MCs (Kohler et al., 2014). In addition, a potent inhibition of serine-proteases such as

thrombin and trypsin has been observed for AG 828A. However, the toxicological effects of AG 828A are not yet sufficiently explored.

Glutathione-S-transferase (gst), a member of the phase II detoxification enzymes, has been proposed to be involved in the detoxification of MCs by conjugating the toxins to glutathione in daphnids (Chen et al., 2005; Ortiz-Rodriguez and Wiegand, 2010; Pflugmacher et al., 1998). Conjugation to glutathione increases the water solubility of a substance and therefore facilitates its excretion (Wiegand and Pflugmacher, 2005). Still, recent findings suggest gst-mediated conjugation to be a general cyanobacterial effect in response to oxidative stress rather than a specific MC effect, therefore being of minor relevance in MC detoxification (Sadler and von Elert, 2014).

The accumulation of vitellogenin takes place under hormonal control and is a key event in ovarian maturation. However, both, the vitellogenin gene (*vtg*) and protein were also found to be strong biomarkers for estrogenic activity. Besides evaluation of estrogenicity, the evaluation of the *vtg* levels might gain insight into the responsiveness of the gene to exposure to environmental toxins as a general detoxification mechanism. The induction of *vtg* was shown in the presence of estrogenic compounds in fish (Folmar et al., 1996; Zhang et al., 2005) and amphibians (Palmer et al., 1998), but also in crustaceans such as barnacle, shrimp and prawn (Billinghurst et al., 2000; Huang et al., 2006; Yano and Hoshino, 2006). In *Daphnia magna*, strong suppression of *vtg* expression was triggered by ecdysteroids (Hannas et al., 2011) and by juvenile hormones and their agonists (Tokishita et al., 2006). However, in contrast to fish, daphnids are predominantly parthenogenetic organisms and thus, the relevance of *vtg* for their reproduction capacity remains unclear.

Recently, effects of different *Planktothrix agardhii* extracts on *D. magna* population dynamics were investigated and severe effects of MC-deficient *Planktothrix* strains on life history parameters of *D. magna* were found that might indicate endocrine disruption (Hulot et al., 2012). It was concluded that metabolites others than MCs must be responsible for the negative effects on *D. magna* reproduction processes. In an earlier study toxic effects of chlorine and sulfate containing aeruginosins that are present in *Planktothrix* strains when MCs are lacking were confirmed (Kohler et al., 2014). Thus, we hypothesize that these compounds might be responsible for the adverse effects on *D. magna*.

Furthermore, exposure of zebrafish to extracts of *Microcystis* but not pure MC-LR resulted in the induction of *vtg* (Rogers et al., 2011), thus indicating an estrogenic activity of cyanobacterial extracts.

The aim of this study was therefore to identify effects of AG 828A, one chlorine and sulfate containing aeruginosin representative, on life history parameters in *D. magna* in comparison to MC-LR. Moreover, comparative gene transcription analyses of MC-LR and AG 828A at two different developmental stages were assessed in *D. magna*. To obtain indications on its toxicological profile, transcriptional analysis of *vtg* was performed based on its reported previous induction by cyanobacterial extracts in fish and *gst* due to its role in metabolism.

2. Materials and methods

2.1 Cyanobacterial toxins

Aeruginosin 828A (AG 828A) was isolated from *Planktothrix rubescens strain* 91/1, grown in 300 mL Erlenmeyer flasks at 20 °C under constant light conditions at an irradiation of 6 µmol m^{-2} s^{-1} from fluorescent tubes (Osram 930; Lumilux Delux; Warm White 3000K) in 120 mL mineral medium (Jüttner et al., 1983). *Planktothrix rubescens* 91/1 cultures were transferred every five to nine weeks, by transferring approx. 20 mL of dense culture to 100 mL of new mineral medium. Neither growth phase nor cell density was determined before harvesting. Cyanobacterial cultures were harvested by centrifugation (25'700 g for 20 min) to obtain a pellet. The supernatant was filtered over a plankton net (22 um mesh size), to harvest remaining filaments. Both, the pellet and the remaining filaments were combined and frozen at -20°C over night. Thawed *cyanobacterial* biomass was extracted twice with 50 % aqueous MeOH (10 mL per g wet weight) for 2 h in the dark. After centrifugation (25'700 g for 15 min), the supernatant was separated by HPLC equipped with a photodiode array detector using a reversed phase column (Hydrosphere C18, YMC, 4.6 x 250 mm, Stagroma, Reinach, CH) under the following conditions: solvent A was UV-treated deionised water (+ 0.05% trifluoroacetic acid; TFA), solvent B: HPLC-grade acetonitrile (+ 0.05% TFA) applying a flow rate of 1 mL min^{-1}. A linear increase in two steps was applied (solvent B from 20 % to 70% in 50 min, 70 to 100% in 2 min, isocratic for additional 10 min). Mass spectra were recorded on a LC-MS (LCQ Duo mass spectrometer, Finningan Thermoquest, USA) equipped with an electrospray ionization source (ESI-MS) prior to isolation. AG 828A *m/z* = 829.3 $[M+H]^+$ eluted under the conditions applied after 16.0 min, as determined by HPLC. Mass accuracy was determined to be -24.5334 ppm. Fractions of 2 min containing the desired peptides were collected for further purification. Purified AG 828A free of isomerization products was obtained by using a C18 cartridge (10 g, 60 mL, Mega Bond Elute, Varian, Agilent Technologies, Basel, CH), which was equilibrated with 10 % aqueous MeOH (ROTISOLV ≥ 99.95%, LC-MS-Grade, Roth, CH). The cartridge was flushed with Nanopure water (180 mL), and AG 828A was eluted with 80 % aqueous MeOH according to (Kohler et al., 2014). Aqueous methanol was removed by a rotary evaporator (Rotavapor, Büchi; 40°C, 35 mbar). The purified peptides were transferred to storage vials (Infochroma; 2ml) by 100% methanol and a dry powder was obtained by removing the solvent by a gentle N_2 flow at

40°C. The described purification procedure was repeated until sufficient purity was achieved. After purification about 1600 µg of pure AG 828A (> 99% HPLC) could be obtained by applying 300 HPLC runs. Pure MC-LR (> 99 % HPLC) was purchased from Enzo Life Sciences (ELS AG, Lausen, Switzerland).

2.2 Daphnia cultures

Daphnia magna strain Iinb1 (kindly provided by Dieter Ebert, University of Basel, Switzerland) was kept at 16:8 hours light dark cycle (20 µmol m^{-2} s^{-1}) in M4 medium (Elendt, 1990) in 1 L glass beakers at 18 °C. Transfer into new medium was conducted every two to three weeks. The animals were fed every second day with *Scenedesmus obliquus* (kindly provided by Dieter Ebert, University of Basel, Switzerland) and *Scenedesmus subspicatus* at non-limiting food concentrations. Both *Scenedesmus* strains were grown in 300 mL Erlenmeyer flasks at 20 °C under constant light conditions at an irradiation of 6 µmol m^{-2} s^{-1} from fluorescent tubes (Osram 930; Lumilux Delux; Warm White 3000K) in 120 mL mineral medium (Jüttner et al., 1983).

2.3 Life-history parameter of Daphnia magna

Two stock solutions were prepared from dried peptides MC-LR and AG 828A in 100% dimethyl sulfoxide (DMSO; from Sigma–Aldrich, FlukaAG, Buchs, Switzerland) of 1 g/L and 10 g/L. Upon medium change, the stock solutions were diluted with M4 medium (containing respective concentrations of food algae) to obtain final nominal concentrations of 100 µg/L and 1000 µg/L MC-LR and AG 828A respectively (0.01% DMSO). Ten new-born daphnids (age <24 h) were exposed to 100 µg/L and 1000 µg/L, respectively, of AG 828A or MC-LR (both dissolved in 0.01% DMSO) for 21 days. Additionally, solvent controls (10 animals) with 0.01% DMSO were included. Individuals were kept separately in 25 mL glass beakers. The exposure medium M4 containing the appropriate cyanobacterial toxin at required concentrations and 0.01% DMSO (solvent control), respectively, were renewed every 48 hours with one exception of a renewal after 72 hours on day 5 at the end of week one. Treatments contained 0.5 x 10^6 cells/ mL of *S. subspicatus* until daphnids reached maturity, afterwards 1 x 10^6 cells/ mL were added. Cell numbers of *S. subspicatus* were quantified by flow cytometry prior to medium renewal.

The size of the animals was determined microscopically with a Leica MZ FL III fluorescence stereomicroscope (Leica Microsystems GmbH) equipped with an AxioCam 105 color camera (Carl Zeiss Microscopy GmbH) at three time points: prior to the experiment, at occurrence of the first batch (maturity) and at the end of the experiment on day 21. Micrographs were analysed with the ZEN image analysis software (ZEN 2012, blue edition, Carl Zeiss Microscopy GmbH). Each animal was inspected daily to monitor moult, appearance of batches, numbers of offspring and survival/immobilisation of the daphnids. The number of offspring was determined every 24 h. The neonates were subsequently transferred into new beakers. The accumulated number of offspring was totalled from the daily collected numbers.

Purified cyanobacterial peptides are very precious, as isolation and purification of cyanobacterial peptides is very laborious and time consuming. Thus, a previous determination of an LC_{50} for D. magna could not be carried out. Instead, previously obtained toxicity data for the crustacean *T. platyurus* were considered and compared to MC-LR toxicity data for *D. magna* (Blom and Jüttner, 2005; Chen et al., 2005; Kohler et al., 2014). Moreover, the 2 concentrations 100 µg/L and 1000 µg/L were chosen in accordance with previous *D. magna* studies with MC-LR, in order to allow for comparison (Chen et al., 2005). Additionally, limited amount of AG 828A allowed only two concentrations for the life-history-analysis, therefore, we decided for a high (1000 µg/L) and a low (100 µg/L) concentration, where no acute toxicity was expected.

Information about peptide concentrations in natural bloom samples are available for MC-LR and are in the range of the tested concentrations (Dyble et al., 2008; Jones and Orr, 1994; Sabart et al., 2010). For AG 828A, these data are not available yet. However, data about cell densities of MC-lacking chemotypes in natural blooms make it very likely, that the tested concentrations may be found in nature (Ostermaier and Kurmayer, 2009).

2.4. Transcription analysis of target genes

A cohort of *D. magna* originating from synchronized mothers (born within 24 h) was kept as described above at 16:8 hours light dark cycle (20 µmol m^{-2}-s^{-1}) in M4 medium (Elendt, 1990) in 1L glass beakers at 18°C for subsequent exposure to two different cyanobacterial peptides. AG 828A and

MC-LR were tested at two different concentrations: 100 µg/L and 1000 µg/L (0.01 % DMSO final concentration); in addition, a control with M4 medium containing 0.01 % DMSO was included. For each treatment, 10 animals were tested individually. Each animal was kept in 1 mL volume in 24 well plates (TPP Techno Plastic Products AG, Switzerland). The animals were exposed to the cyanobacterial toxins for 24 h. *D. magna* were fed the green alga *S. obliquus* at a concentration of 1 x 10^6 cells /mL, as determined by flow cytometry. The experiment was conducted once with five days old e.g. juvenile *D. magna* and once with 10 days old e.g. mature individuals, each with eggs in the brood pouch (10 individuals per dose group each). The exposure was terminated by pooling the individually exposed daphnids in RNAlater® Stabilization Solution (Ambion®, Life Technologies, Thermo Fisher Scientific) and kept at -23°C until RNA extraction.

2.5. RNA extraction, reverse transcription and RT-q-PCR

Total RNA was isolated from pools of ten *D. magna* using RNeasy Mini Kit (Qiagen, Basel, Switzerland). RNA purity was measured by Qubit® (Invitrogen™) and RNA integrity was controlled using an Agilent 2100 Bioanalyzer and Agilent 6000 Nano kit (Agilent Technologies, Basel, Switzerland). RNA extraction, quantification and quality control was performed in collaboration with the Genetic Diversity Centre (GDC), a technology platform of the Department of Environmental Sciences at the Swiss Federal Institute of Technology Zürich. cDNA was produced by reverse transcription of 250 ng RNA by Moloney murine leukemia virus reverse transcriptase (Promega Biosciences, Inc., Wallisellen, Switzerland) with random hexamers (Roche, Rotkreuz, Switzerland) and deoxynucleoside triphosphate (Invitrogen™). The reaction mixture was incubated for 5 min at 70 °C and then for 1 h at 37 °C. The reaction was terminated by heating to 95 °C for 5 min.

Real-time quantitative reverse transcription polymerase chain reaction (RT-qPCR) analysis of selected target genes including vitellogenin (*vtg*) and glutathione-S-transferase (*gst*) was performed based on SYBR green fluorescence (SYBR green PCR master mix; Roche). The gene encoding actin was used as internal standard (housekeeping gene; (Schwarzenberger et al., 2010)). Gene specific primers were obtained from published sequences (Supplementary Material, Table S1). The amplification conditions were 95 °C for 5 min for initial denaturing, 40 cycles of 95 °C for 30 s for denaturing, 60 °C

for 60 s for annealing and elongation. Subsequently, a melting curve was conducted. All reactions were done in triplicate using a BioRad real time PCR machine (CFX 96 Real Time System). Negative controls with H_20 were conducted for each primer pair as well as noRT-controls without reverse transcriptase to check for remaining gDNA in the samples.

2.6. Chemical analysis of exposure medium

For the life history analysis, cyanobacterial peptide concentrations in the exposure medium were determined prior to the experiment (t = 0 h), and before every medium change (72 h exposure) by HPLC. For quantification, peptides in 10 mL of the exposure medium were concentrated over C18 cartridges (10 g, 60 mL, Mega Bond Elute, Varian, Agilent Technologies, Basel, CH), and eluted from the C18 cartridge with 100 % MeOH. The solvent was then evaporated by rotary evaporator (Rotavapor, Büchi; 40°C, 35 mbar). The remaining dry powder was then re-dissolved in 4 mL 100% methanol, continuously transferred to HPLC vials (Infochroma; 2ml) and brought to dryness by gentle N_2 flow at 40°C. The dry powder was re-dissolved in 400 mL 60% aqueous methanol and thereof 200 mL was applied for HPLC measurement. Single measurement was performed. For the quantification procedure, calibration curves for AG 828A and MC-LR were established using prepared standard solutions in the range of 1 and 10 µg mL^{-1}. The calibration curves were based on the peak area recorded at a wavelength of 220 nm (AG 828A) and 239 nm (MC-LR). Cyanobacterial peptides were measured by HPLC with two solvents: solvent A was UV-H_2O + 0.05 % TFA and solvent B was acetonitrile + 0.05 % TFA. AG 828A was quantified using the gradients described above: a linear increase in two steps was applied (solvent B from 20 % to 70% in 50 min, 70 to 100% in 2 min, isocratic for additional 10 min). MC-LR was quantified using a gradient with a linear increase in two steps (solvent B from 35% to 70% in 30 min, 70% to 100% in 2 min). At t = 0 h, the following concentrations have been determined: MC-LR: 95 ± 4.5 µg/L and 945 ± 33 µg/L; AG 828A: 105 ± 10 µg/L and 990 ± 105 µg/L. Before changing the medium, the following concentrations have been determined: AG 828A: 82 ± 5 µg/L and 948 ± 56 µg/L; MC-LR: 82 ± 51 µg/L and 922 ± 99 µg/L. The given values are means of all medium renewals.

For the transcriptional analysis, exposure concentrations of cyanobacterial peptides were determined prior to exposure (t = 0 h) with HPLC. Peptides were concentrated as described above, using

the gradients described above. At t = 0 h, the following concentrations have been determined: MC-LR: 106 ± 20 µg/L and 966 ± 74 µg/L; AG 828A: 78 ± 6 µg/L and 913 ± 27 µg/L. Because of the little volume used in the assays, a reliable quantification of the concentrations after 24 h could not be carried out.

2.7. Statistics

For the survival curves, the log-rank (Mantel-Cox) Test was applied using GraphPad Prism 5 (GraphPad Software, San Diego, USA). For the life history analysis, statistical testing for significant differences between the treatments and the solvent control was performed for the day of 1st egg appearance, the day of 1st clutch, and the increase in body size until maturity (Fig. 2). Additionally, differences in the mean number of eggs per clutch (Fig. 3), and in the accumulated number of offspring per female (Fig. 4) were determined. Each data point represents one individually kept animal. All calculations were performed by one way analysis of variance (1-way ANOVA) followed by a Dunnett's post hoc test (95% confidence interval) using GraphPad Prism 5 (GraphPad Software, San Diego, USA). Results are shown as mean ± 1 standard deviation. Differences were considered significant when $P \leq 0.05$. In some cases, the variability in some treatments is comparably high. This might be related to the comparatively small samples size (10 individuals).

For the transcriptional analysis, the raw data were normalized and log2-transformed. Each data point represents the pooled RNA from 10 individually exposed animals. Genes with a mean fold change (log2) ≥ 1 and ≤ -1 were considered as differentially expressed genes.

3. Results

3.1 Life history parameters of Daphnia magna

The survival rate of *D. magna* after 21 days was 80% in the solvent control and 90% at 100 µg/L MC-LR. A decreased survival was detected at both exposure concentrations of AG 828A (60% and 50%, respectively). The lowest survival (40%) was found at 1000 µg/L MC-LR (Fig. 1). Mortality was highest between days 6 and 9, suggesting a higher sensitivity of juvenile animals. At 1000 µg/L MC-LR and AG 828A survival in aged daphnids was negatively affected at the late phase of the experiment (Fig. 1). The log-rank (Mantel-Cox) test showed no significant differences between the survival curves. Median survival time values (LT_{50}) could be only determined for AG 828A and MC-LR at 1000 µg/L and were found to be 20.5 and 14 days, respectively.

The time to first eggs was delayed by about 2.4 days in *D. magna* exposed to high concentration of AG 828A, as compared to the control animals (Fig. 2, left panel). Albeit not significantly different, the low probability value ($p = 0.0613$) might indicate an effect of AG 828A on the time of egg production. AG 828A also increased the mean time to first clutch from 13.5 (controls) to 14.3 days in *D. magna* exposed to 1000 µg/L (Fig 2, middle panel). Moreover, the presence of 1000 µg/L AG 828A resulted in significantly increased body length at time of maturity as compared to the initial size (Fig. 2, right panel).

The mean number of clutches per female *D. magna* in the control treatment was 3.5 during the 21-day experiment. While the number in all other treatments were comparable, the mean number of total clutches per daphnids decreased to 2.6 at the highest AG 828A concentration. Significant differences were found for the number of eggs that were produced in the first clutch (Fig.3). One female in each AG 828A treatment developed one egg (first clutch) in the brood pouch that degenerated. AG 828A treatments resulted in female daphnids not capable of producing a second and third clutch. The accumulated number of offspring was significantly lower at 1000 µg/L AG 828A (Fig. 4).

During the chronic exposure of 21 days, moulting disruption in the daphnids of all treatments were investigated. These animals were unable or had difficulties to shed the old integument although a new one had already been produced (Supplement material, Fig. S1, left panel). In the control as well as in the low MC treatment, moulting disruption was not observed. Only one daphnid exposed to 1000

µg/L died from these moulting difficulties, on day 19. However, most daphnids exposed to AG 828A showed severe signs of moulting disruption. Two animals exposed to 100 µg/L died from moulting disruption (on days 6 and 8), one daphnid showed severe signs, but recovered completely. Most of the animals exposed 1000 µg/L AG 828A suffered from prolonged and complicated moulting procedure. Five of these animals with moulting disruption died (two on days 6, one on day8, 10, and 20). One daphnid showed symptoms, but survived, and never did produce any offspring. Only in the treatments with AG 828A, the impaired moulting was accompanied by a deformation of the brood pouch (Supplement material, Fig. S1, right panel). Additionally, the body surface of these daphnids was rapidly covered by food particles.

3.2 Transcriptional chances of target genes

Selected target genes were investigated in the homogenated body tissues of *D. magna*. All mRNA alterations were compared to the solvent control (0.01% DMSO). The transcripts of *gst* were not differentially expressed in 5 days and 10 days old daphnids after exposure to both concentrations of the two cyanobacterial peptides for 24 h (Fig. 5, left panel).

A down-regulation of the *vtg* transcripts was observed for 5 day old daphnids, either when exposed to lower concentrations of AG 828A, or when exposed to both cyanobacterial peptides at the higher concentrations (Fig 1, right panel). In contrast, exposure of mature *D. magna* at the age of 10 days did not result in altered *vtg* transcript levels, independent of the concentration and the cyanobacterial toxin tested (Fig. 5, right panel).

4. Discussion

4.1. Toxicity of MC-LR and AG 828A

Exposure of *D. magna* to high concentrations of AG 828A resulted in significantly lower reproduction. Moreover, our data indicate negative effects on survival, moulting abilities and normal development. Acute toxicity to *Daphnia* of 24 h - LC_{50} in the range of between 10.7 and over 50 µg mL^{-1} for MC-LR have previously been reported (DeMott et al., 1991). The chronic toxicity determined in our present study is similar as previously described with a survival of 100 % when exposed to 100 µg/L MC-LR and 50 % when exposed to 1000 µg L^{-1} (Chen et al., 2005). The present experiments also showed that both concentrations of AG 828A severely decreased the survival of daphnids after 21 days, which was not expected for the low concentration of the toxin (Fig. 1). Comparing reproduction success, somatic growth and moulting difficulties, we suggest these toxins to act on different cellular targets and to have different mode of actions. The cell numbers of the food algae we used in our study was rather low, and possibly the combined effect of food limitation and toxins might have increased mortality between day 6 and 9. Since almost no mortality was found in the control treatment during this period, it is, however unlikely that food limitation was the main cause of the mortality observed in the other treatments.

4.2 Life history characteristics of **D. magna**

The adverse effects of MCs and *Microcystis aeruginosa* on body length, time to first clutch, clutch numbers, and population growth of *D. magna* are well known. Dose-depended impaired reproduction was reported, as well as reduced survival (Chen et al., 2005). Recently, also the effects of different *Planktothrix agardhii* extracts on *D. magna* population dynamics were investigated (Hulot et al., 2012). These authors concluded that additional, unknown metabolites must be responsible for the negative effects on *D. magna* reproduction processes, as also *Planktothrix* strains not capable of producing MCs, affected life history traits. Moreover, the authors noticed that such negative effects on reproduction function are typically observed for endocrine-disruptive compounds (Hulot et al., 2012).

We therefore examined to what extent aeruginosin AG 828A, isolated from a MC-deficient *Planktothrix* strain, negatively affects the life history parameters in *D. magna* in comparison to MC-LR.

The key parameters in examining life history traits in daphnids are the allocation of resources to growth and reproduction (Ebert, 1994). If the environment becomes less favourable, the age at maturity increases and the size decreases (Ebert, 1994), as shown for *D. magna* treated with different concentrations of nitrite (Xiang et al., 2012). In our study, both treatments with MC-LR did not affect these parameters significantly. However, AG 828A affected the age at maturation and the increase in body length at time of maturity (Fig. 2). The animals required more time to reach maturation, and the somatic growth increased. At the same time, AG 828A significantly lowered the reproduction success of. *D. magna*, while the egg production in the MC-LR treatments was not reduced (Figs. 3 and 4). We suggest that daphnids exposed to high concentrations of AG 828A allocated their resources rather into somatic growth than into reproduction. However, a mean value of 4.5 eggs per clutch is very low compared to earlier findings in the literature (Coors and De Meester, 2008; Ebert, 1991). The presence of additional stressors, such as food limitation could explain the low offspring number. While it may be considered problematic to introduce more than a single stressing factor for the experimental assessment of toxicity, such food limitation might in fact better mimic the in situ situation of daphnids than unlimited supply. Food is one of the most important constrains for zooplankton growth in lakes; easily ingestible phytoplankton biomass decreases in lakes during early summer, while grazing resistant taxa with inferior food quality increases (Ferrao et al., 2003). Under these conditions, secondary metabolites such as AG 828A might be even more harmful to daphnids and other zooplankton.

AG 828A is an inhibitor of serine proteases such as trypsin (Kohler et al., 2014). As a consequence of exposure, essential food might not be readily available for the daphnids. It has been previously reported that low algal concentrations led to delayed age at initial reproduction and to decreased reproduction in *D. magna*, but it was accompanied by a decreased body growth (Porter et al., 1983). This was also found in *D. magna* fed with *M. aeruginosa* (Liu et al., 2011). In that study, the animals were slightly older but significantly smaller at maturity. Low food quality usually limits zooplankton growth (Müller-Navarra et al., 2000; Wacker and von Elert, 2001), while, at the same time, it decreases the number of offspring (Enserink et al., 1995; Pajk et al., 2012). However, since animals

exposed to AGA 828a increased in body size compared to the controls, protease inhibition does not seem to be the primary mode of action of this compound.

Recent findings of endocrine-disrupting effects of anthropogenic chemicals have raised concerns about their possible ecological impact. Long-term steroid exposure reduced *D. magna* fecundity and fertility (Barbosa et al., 2008). Such effects were also found in *D. magna* in the presence of different pesticides, resulting in increased time to first brood and decreased offspring (Kim et al., 2008). Moreover, toxicity, delayed time to first brood, and the number of offspring of *D. magna* exposed to mefenamic acid was accompanied by an increase in body growth (Collard et al., 2013). Exposure of *D. magna* to fenoxycarb, a model juvenile homologue, led to developmental abnormalities such as poorly developed antennae and spines, but also swollen brood chambers (Kim et al., 2011). The latter malformation was repeatedly observed in daphnids treated with AG 828A (Fig. S1). Collectively, our data suggest a similar adverse effect of AG 828A in *D. magna* as found for these compounds with endocrine-disrupting properties. However, these are preliminary data that need further confirmation. Moreover, this similarity of symptoms in *D. magna* does not serve as proof that endocrine disruption was caused by AG 828A. Elucidation of the mode of action of AG 828A remains to be done for clarification.

4.3 Moulting disruption

Our findings indicate that AG 828A could lead to lethal moulting disruption in *D. magna*. Microviridin J, isolated from *Microcystis* strain UWOCC MRC, also caused moulting disruption in *D. pulicaria* (Rohrlack et al., 2004). The authors described a similar pattern, e.g. that the daphnids were not able to shed the old integument or that parts of the old integument stayed attached and thus prevented the branches of the antennae from unfolding in a normal way. In addition, we repeatedly observed a swollen brood pouch, which was not reported for microviridin J. Structurally both toxins, AG 828A and microviridin J, are quite different. AG 828A is a short linear peptide containing a chlorine and a sulfate moiety , whereas microviridin J is a large multicyclic peptide (Rohrlack et al., 2003). However, both are strong inhibitors of trypsin-like enzymes (Kohler et al., 2014; Rohrlack et al., 2003). Two explanations have been suggested for the moulting disruption effect of microviridin J; incomplete protein digestion

resulting in a depletion of amino acids essential for production, and sclerotization of a new integument and a possible inhibition of proteases that normally digest the integument to be replaced (Rohrlack et al., 2004). Both toxins, AG 828A and microviridin J, might share a similar property in this respect.

4.4 Glutathione-S-transferase

It was proposed that detoxification in daphnids takes place by conjugation of the toxin with glutathione by gst (Pflugmacher et al., 1998). In our study, *gst* was chosen as a possible marker for the detoxification process in *D. magna*. However, *gst* levels in daphnids exposed to MC-LR, and AG 828A were not differentially expressed (Fig 5). The age of the daphnids may strongly affect this response. Earlier reports showed a positive effect on *Daphnia* gst activity after direct MC-LR exposure for juvenile daphnids (< 3 days) (Chen et al., 2005; Ortiz-Rodriguez and Wiegand, 2010). Long exposure time to MC-LR of 48 h was necessary to induce a significant increase in the gst level in adult daphnids (7 days); after 24 h, only a slight decreasing tendency compared to control was detected in aged daphnids (Ortiz-Rodriguez and Wiegand, 2010). Although the formation of MC-glutathione products *in vivo* was shown for a variety of aquatic organisms (Li et al., 2014; Zhang et al., 2009; Zhang et al., 2012), the evidence for such conjugates in daphnids is still missing. A recent study revealed that a diet of MC-containing *Microcystis aeruginosa* strain PCC7806 cells or of its MC-deficient mutant did not result in differences between the (increased) gst enzyme levels of daphnids (Sadler and von Elert, 2014). The authors argued that such an increase in gst activity presumably indicates an involvement of gst in a general oxidative stress response rather than a direct detoxification of MCs, and that the role of gst might even obscure the MC detoxification effect. Future studies are required to investigate whether detoxification in daphnids occurs via gst and whether unaltered *gst* transcript levels also imply no changes in gst activity.

4.5 Vitellogenin

Recent progress in genomics has facilitated the identification of potential biomarkers including *vtg* to determine endocrine disrupting effects in *Daphnia* (Kim et al., 2011). Vitellogenin (vtg) is essential as a feeding reserve for the developing embryo, and thus necessary for normal development in daphnids (Kim et al., 2011). It has become increasingly evident that the function of vtg appears to

extend beyond this nutritional role. Vtg was able to reduce oxidative stress by scavenging free radicals in honeybees (Seehuus et al., 2006), and a similar antioxidant activity was also found in *Caenorhabditis elegans* (Nakamura et al., 1999). Moreover, *vtg* has been well known as a reproduction related gene in fish that is affected by endocrine disruption (Henry et al., 2009). Recently, it was suggested that possible interactions may exist between the immune and endocrine system and that vtg might contribute to the immune-competence in aquatic organisms (Gelinas et al., 2013). Alterations in *vtg* levels have been described for xenoestrogens in crustaceans albeit with inconclusive results (Matozzo et al., 2008). However, various juvenile homologues and their agonists (such as pyriproxyfen, fenoxycarb, methoprene and methyl farnesoate) induced a suppression of *vtg* transcripts in *D. magna* (Kim et al., 2011; Tokishita et al., 2006).

In our study, the effect of AG 828A on *vtg* transcriptional changes were analysed in juvenile and adult daphnids and compared to those of MC-LR. Both oligopeptides led to a decrease in *vtg* transcripts in juveniles (5 days) but not adult daphnids. The interpretation of the down-regulation of *vtg* remains difficult. On the one hand, the *vtg* down-regulation may be regarded as an antiestrogenic effect. However, earlier studies have shown that *Microcystis* extracts but not pure MC-LR led to up-regulation of *vtg* in zebrafish (Rogers et al., 2011). On the other hand, the down-regulation of *vtg* may suggest a reduced fitness in the juvenile daphnids. Age-related differences in the *vtg* expression were previously found in these organisms. While a suppression of *vtg* transcript levels through juvenoids was reported for juvenile, reproductive naïve daphnids (Tokishita et al., 2006), no effect of juvenoids were observed with moult-synchronized adult animals that had experienced a first reproduction/ moult cycle (Hannas et al., 2011). Down-regulation of *vtg* transcripts caused by AG 828A was found in juvenile daphnids. *Vtg* expression is similarly altered through MC-LR exposure although this toxin is not known to interfere with the endocrine system. Therefore, altered *vtg* levels may be an indication for general toxicity in daphnia rather than specific developmental impairments. However, the mechanisms responsible for the elevation or suppression of *vtg* are not well understood and our preliminary data need further confirmation.

5. Conclusion

This study provides novel information about adverse effects of AG 828A on *D. magna*, which is characterized by moulting disruption and mortality. Long-term exposure of daphnids to this toxin showed a delay of the age at maturation accompanied by an increase in body length at time of maturity, as compared to the initial size. AG 828A significantly lowered the reproduction success of. *D. magna* and reduced the *vtg* transcript in juveniles, which might be interpreted as an endocrine effect.

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Appendix A. Supplementary Data

Supplementary material related to this article can be found, in the online version, at http:// http://www.journals.elsevier.com/aquatic-toxicology/.

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Figures and Legends

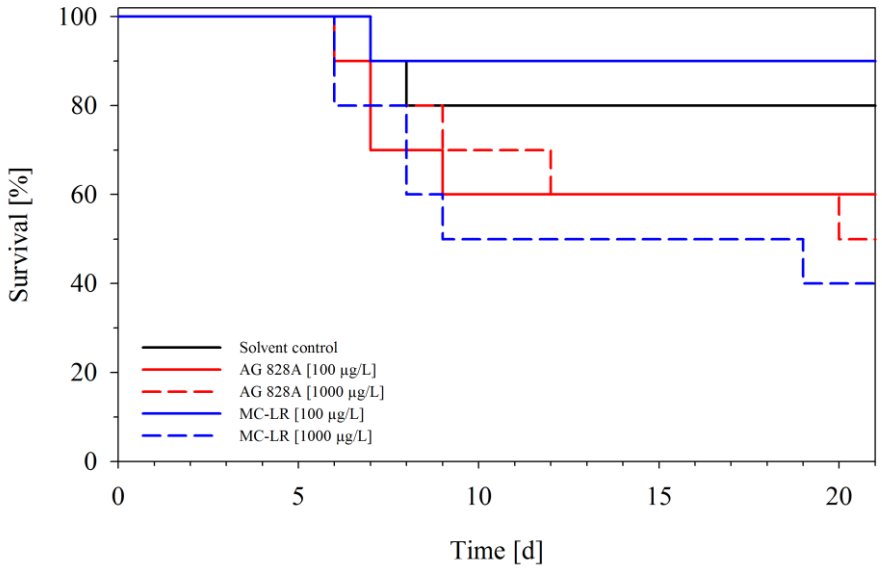

Fig. 1 Survival [%] of *D. magna* in the solvent control (black line) and at exposure to 100 µg/L (solid lines) and 1000 µg/L (dashed lines) of AG 828A (red) and MC-LR (blue).

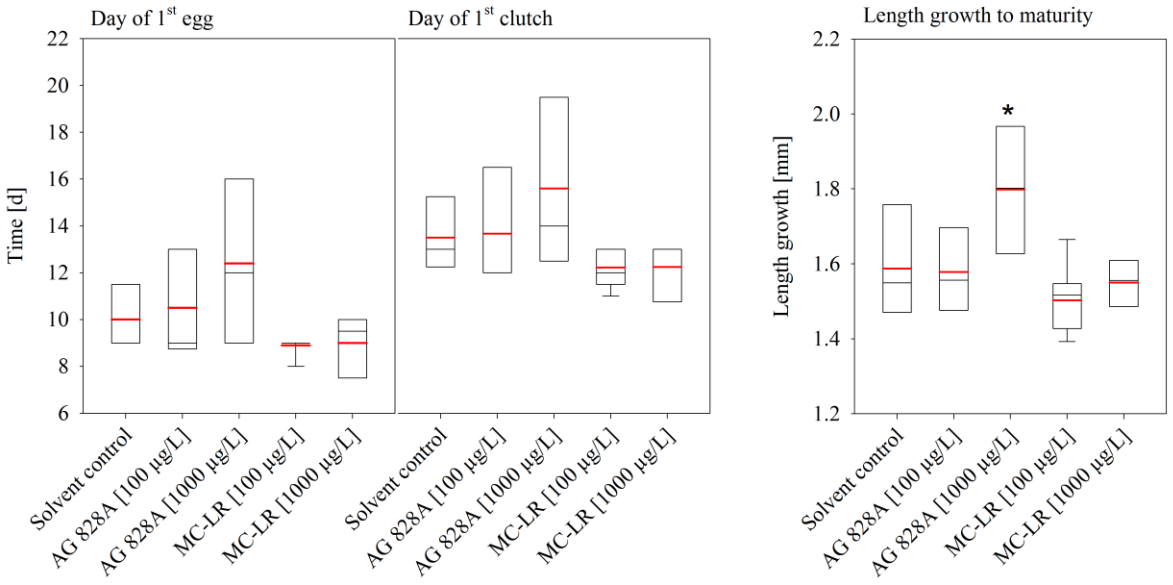

Fig. 2: Day of first batch of eggs, day of first clutch and length growth of *D. magna* in solvent control and exposures to 100 and 1000 µg/L of AG 828A and MC-LR. Length growth refers to differences in body size at the time of maturity as compared to the initial body size. Black lines indicate median, red lines indicate mean values. Asterisks indicate a significant difference to solvent control (* < 0.05).

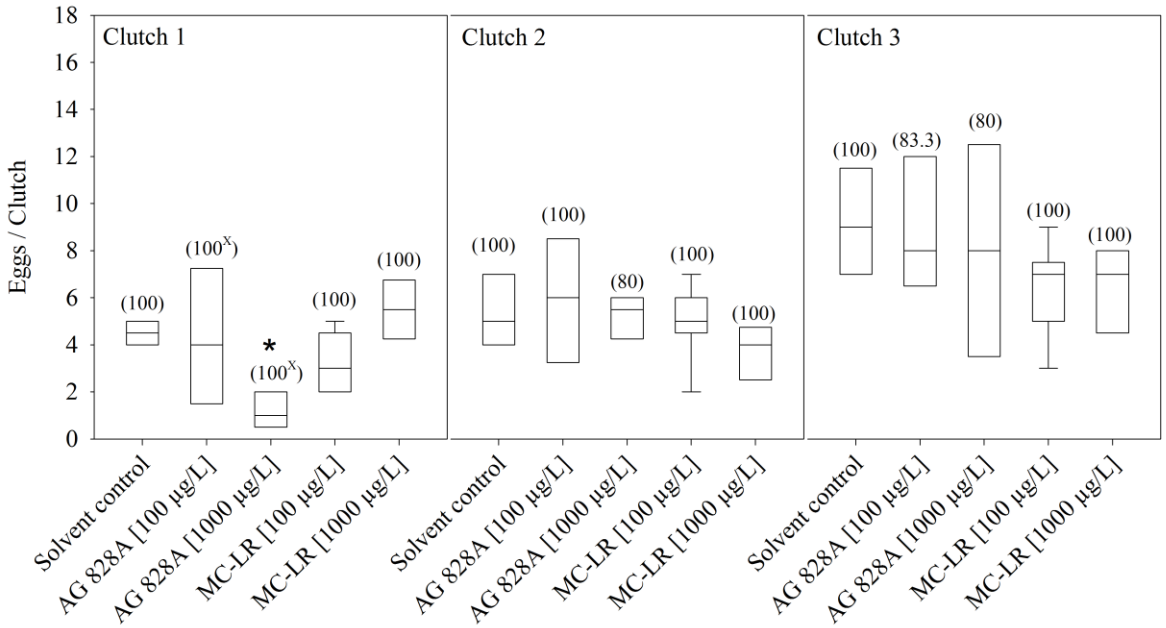

Fig. 3: Mean number of eggs per clutch of *D. magna* in the control treatment or of animals that were exposed to 100 and 1000 µg/L of AG 828A and MC-LR. Numbers in brackets refer to the percentage of animals that produced eggs. ^{X} indicates that some *D. magna* produced eggs that degenerated and did not lead to offspring. Black lines indicate median values. Asterisks indicate a significant difference to solvent control (* < 0.05).

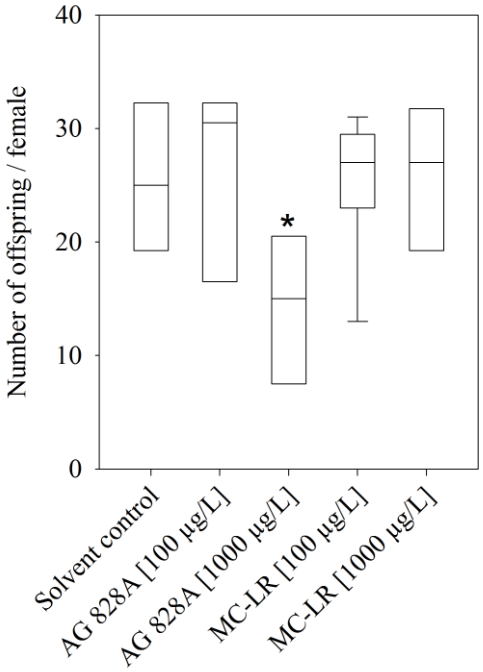

Fig. 4: Accumulated number of offspring per female *D. magna* in the solvent control and after being exposed to 100 and 1000 µg/L of AG 828A and MC-LR for a period of 21 days. Black lines indicate median values. Asterisks indicate a significant difference to solvent control (* < 0.05).

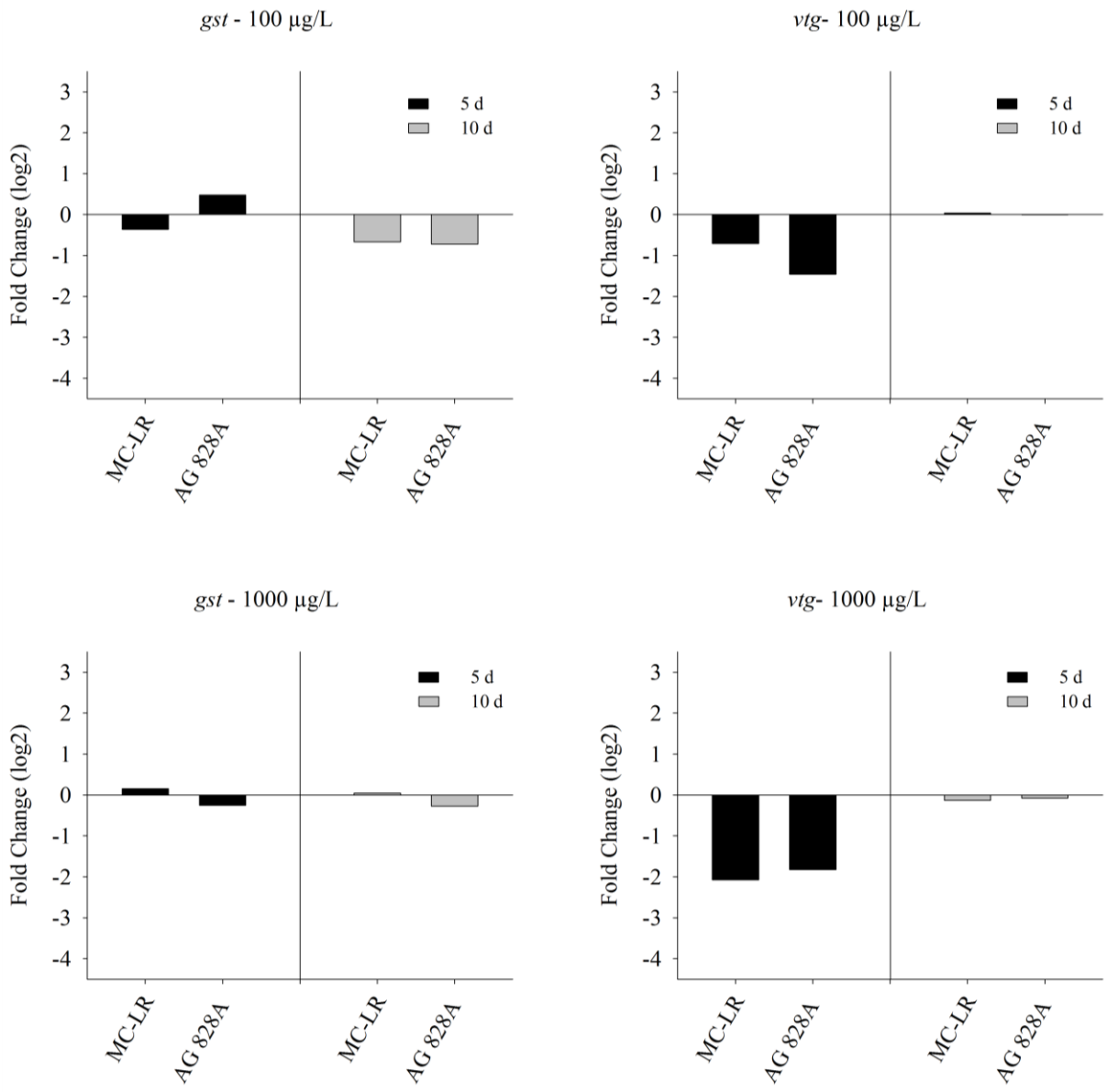

Fig. 5. Relative gene expression in *D. magna* at the age of 5 or 10 days after 24 h exposure to low and high concentrations of MC-LR and AG 828A. All expression levels are referred to solvent control (0.01 % DMSO) and are expressed as fold change (log2). Transcripts of the following target genes are shown: glutathione-S-transferase (*gst*, left panel) and vitellogenin (*vtg*, right panel). Data from pools of 10 daphnia in each dose group.

Supplementary Material

Tables and Figures

Table S1: Primers

Gene name	Accession No	Primer forward 5'-3'	Primer reverse 5'-3'
Actin[1]	AJ292554	CCACAC-TGTCCCCATTTATGAA	CGCGACCAGCCAAATCC
Vitellogenin[2]		AGCGAATCCTACACCA	CGACGAAGCTCAGCAA
Glutathion-S-Transferase[3]		TCAGGCTGG GTTGAGTTTG	GAGCAAGCATTTGTCCATCA

[1] Heckmann et al. 2006

[2] Kim et al. 2011

[3] Poynton et al. 2011

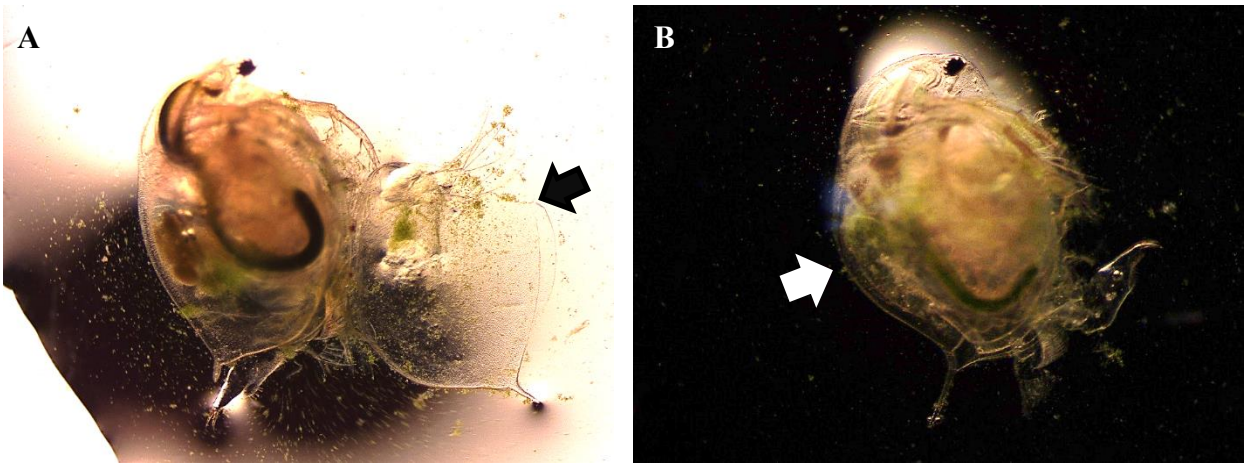

Fig. S1: Malformations caused by 1000 µg/L AG 828A: (A) Impaired moulting, and (B) swollen brood pouch.

DISCUSSION

Specific aspects of cyanobacterial impact on humans and aquatic ecosystems were addressed by the here presented thesis, focussing on potentially toxic oligopeptides produced by cyanobacteria. A few beneficial and mainly harmful effects have been associated with cyanobacteria and their secondary metabolites (Chlipala et al, 2011; Chorus, 1999). Thus, cyanobacterial peptides are regarded as a hot topic in the field of toxicology. In particular, the MCs have opened a large research field due to the frequent MC-related intoxications and fatalities in humans and animals (Campos & Vasconcelos, 2010). However, so far the effects of cyanobacterial secondary metabolites have mainly been addressed from a human und less from an ecosystem perspective. Therefore, cyanobacterial secondary metabolites are often grouped into "cyanotoxins", e.g. microcystins (MCs) and "other bioactive peptides", e.g. serine protease inhibitors (Agha & Quesada, 2014). This artificial differentiation reflects a rather anthropocentric interpretation of the concept of "toxicity". Bioactive cyanobacterial compounds are screened regarding their potential for drug discovery (Singh et al, 2005), but might at the same time be classified as toxins depending on the target organism. Therefore, toxicity in general should be regarded as a relative concept and, most importantly, ecotoxicity must be also part of the discussion.

6. TOXICOLOGICAL AND ECOTOXICOLOGICAL RELEVANCE OF CYANOBACTERIAL PEPTIDES

There is no general "toxicity" of a compound to all organisms, but rather distinct toxic effects of a compound for a particular organism. Moreover, as already stated by *Paracelsus* (Theophrastus Bombastus von Hohenheim, 1493-1541), "dosis sola facit venenum" (the dose makes the poison), i.e., toxicity clearly depends on the quantity of the compound, mode of exposure and sensitivity of the target organism. Toxicity of compounds was and still is mainly assessed in mammalian test organisms like mice and rats. These organisms are small and easy to maintain, and they are well-studied model organisms not only in the field of toxicology, but also in biology in general. However, mice were not found to be susceptible to cyanobacterial serine protease inhibitors such as oscillapeptin J (Blom et al, 2006). Therefore, microviridins, cyanopeptolins, aerucyclamides and aeruginosins that are widely distributed in *Microcystis* and *Planktothrix* strains were long not considered as toxins (**MANUSCRIPT II-IV**).

Standardized ecotoxicological tests are only required for the authorization of newly designed chemicals that are potentially released into the environment and not for secondary metabolites of naturally occurring organisms. However, effects of toxic cyanobacterial peptides on aquatic ecosystems may be considerable, as their concentrations in the water after a bloom collapse can be very high

(Codd, 2000; Zurawell et al, 2005). Still, cyanobacterial peptides other than MCs with focus on ecotoxicological questions have only recently started to gain attention. Consequently, despite the fact that cyanobacteria are important aquatic organisms, our knowledge about the influences of their peptides on aquatic ecosystems is still incomprehensive. Yet, we find very pronounced differences of the effects of cyanobacteria on mammals, crustacean, fish, protists, plants and microorganisms (Blom et al, 2006; Briand et al, 2003; Codd, 2000; Horňák et al, 2008; Leflaive & Ten-Hage, 2007; Oberemm et al, 1999). Aquatic organisms go different ways in order to cope with toxic cyanobacteria. Especially crustaceans are known for their avoidance strategies, but also for their ability to adapt to cyanobacterial toxins (Blom et al, 2006; Kurmayer & Jüttner, 1999; Schwarzenberger et al, 2014). Moreover, some protists have been described to be insusceptible to cyanobacterial toxins, as they preferentially feed on them, e.g. ciliates and amoeba, who form symbioses with MC-degrading bacteria (Blom et al, 2006; Combes et al, 2013; Dirren et al, 2014).

Thus, it is difficult to predict a general toxicity or ecotoxicity of cyanobacterial compounds. Structure-activity relationship (SAR) analyses were suggested to be adapted also for ecotoxicological concerns as they were proven to be useful for drug discovery before (Nagarajan et al, 2013). In order to apply structure-activity assessment, first, the chemical structure of a bioactive or even toxic compound has to be elucidated. Bioassay-guided fractionation assays in combination with high-performance liquid chromatography (HPLC), liquid chromatography-mass spectrometry (LC-MS), and gas chromatography-mass spectrometry (GC-MS) are powerful tools in terms of giving us hints about structural characteristics of possible bioactive compounds (Blom et al, 2010). We applied this technique in **MANUSCRIPT II**, and the chemical analyses of the cyanobacterial extracts revealed the presence of peptides with distinct chemical patterns, e.g. characteristic chlorine isotope pattern and quasi-molecular ions indicating the presence of sulfate in strains that are not able to produce MCs (Table 1 & Figure 7,8).

Table 1. *Planktothrix* culture collection.

STRAINS	*P. AGARDHII* 79	*P. AGARDHII* 364	*P. AGARDHII* 829	*P. RUBESCENS* 21-	*P. RUBESCENS* 550	*P. RUBESCENS* 91/1
PIGMENTATION	GREEN	GREEN	GREEN	RED	RED	RED
MCY GENE CLUSTER	INTACT	DELETION	DELETION	INTACT	INSERTION	INSERTION
MC-PRODUCTION	X			X		
CL-&SO_3-AG PRODUCTION		X	X		X	X

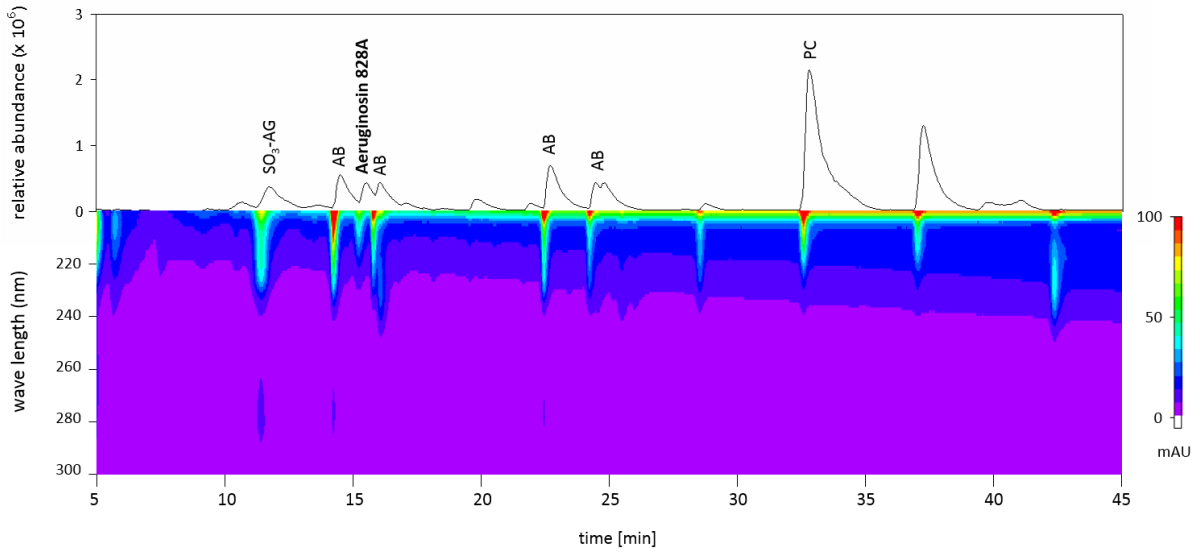

Figure 7. LC-MS data of 50% methanoic extract of *Planktothrix rubescens* 91/1. Chromatogram of absorption (lower panel) and masses (upper panel) of *Planktothrix rubescens* 91/1 extract detected by LC-ESI-MS (liquid chromatography coupled to electrospray ionization mass spectrometry). Aeruginosin 828A eluted after 16 min. [SO_3-AG, sulfated aeruginosin; AB, anabaenopeptin; PC, planktocyclin]

These peptides exhibited toxicity comparable to the one of MCs against sensitive crustaceans. However, structure elucidations of bioactive compounds still require high amounts of purified substances, which is costly in terms of labour and time. Thus, the structure of many bioactive compounds with high relevance for their environment e.g. toxins or infochemicals, remain to be elucidated.

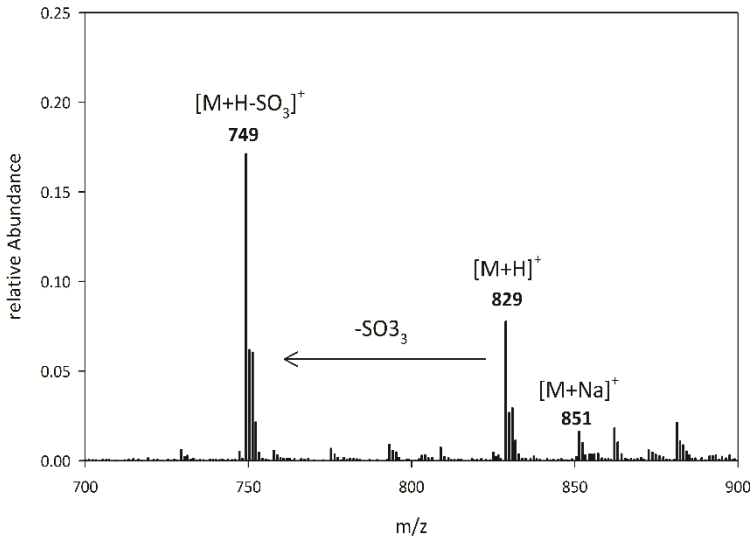

Figure 8. Mass spectra of aeruginosin 828A.

6.1. Removal Of Microcystins From Drinking Water

The symptoms and effects of MCs on humans are divers and have been well described, and no complete picture of all the molecular mechanisms, signalling pathways, cellular targets and modes of action involved in MCs toxicity is yet available (Campos & Vasconcelos, 2010). Moreover, there are great differences in toxicity of the respective MC derivatives, also depending on the organisms tested

(Blom & Jüttner, 2005; Chorus, 1999). Knowledge regarding detoxification mechanisms leading to specific treatment possibilities of intoxicated patients is limited. However, MCs were suggested to be metabolized by conjugation with glutathione, which may lead to higher water solubility and consequently facilitate excretion of MCs (Campos & Vasconcelos, 2010; Pflugmacher et al, 1998). However, in cyanobacterial blooms, bioactive compounds other than MCs may be present. Some of these compounds, e.g. neurotoxic anatoxins and saxitoxins, serine protease inhibitors such as microviridins, cyanopeptolins and aeruginosins or yet unknown substances, may cause adverse effects in humans and animals (Codd, 2000; Nagarajan et al, 2013; Nakamura et al, 1999). MC-toxicity might even be enhanced by these additional cyanobacterial compounds. For example, a tumor-promoting activity of MCs for humans at chronic exposure to very low concentrations of additional cyanobacterial compounds was proposed (Ueno et al, 1996). Thus, the intake of a mixture of cyanobacterial toxins may increase the risk of severe poisonings (Freitas et al, 2014; Koreiviene et al, 2014). Therefore, prevention from intoxication via consumables is crucial. Above all, drinking water purification is pivotal for human health (WHO, 2011). Successful removal of MCs from drinking water is achieved by ozonation (Merel et al, 2013). In developing and threshold countries however, removal of cyanobacterial compounds in drinking water is still a difficult task despite the many different treatments that have been suggested. While a prevention of extensive cyanobacterial blooms would be ideal, several treatments aiming for disruption of harmful algae blooms have been recommended (Fan et al, 2013). However, most of the proposed treatments result in cell lysis, which is all but desirable because it would release the intracellularly stored toxins into the surrounding waters. Subsequent increase of toxin concentrations in waters may be considerable (Jones & Orr, 1994). Recent research has focused on bloom-preventive treatments of waters by applying allelochemicals that inhibit cyanobacterial growth (Laue et al, 2014; Xiao et al, 2014; Ye et al, 2014; Zhang et al, 2014). Still, application of allelochemicals is only a treatment of symptoms, and the underlying problem of eutrophication remains to be addressed: reduction of the anthropogenic nutrient input into waters by appropriate waste water treatment must be established globally.

More and more bacteria are found with the capability to degrade the very stable MCs with specialized enzymes (Dziga et al, 2013). Degradation in three steps was proposed (Figure 9), including linearization, tetrapeptide formation, and further amino acid splitting (Bourne et al, 1996; Ho et al, 2010; Imanishi et al, 2005; Jiang et al, 2011). In fact, MCs are considered valuable substrates as they may contain a wide variety of amino acids, readily available for uptake following MC-degradation. However, degradation of MCs is still not fully understood (Ma et al, 2014; Rastogi et al, 2014; Roegner et al, 2014).

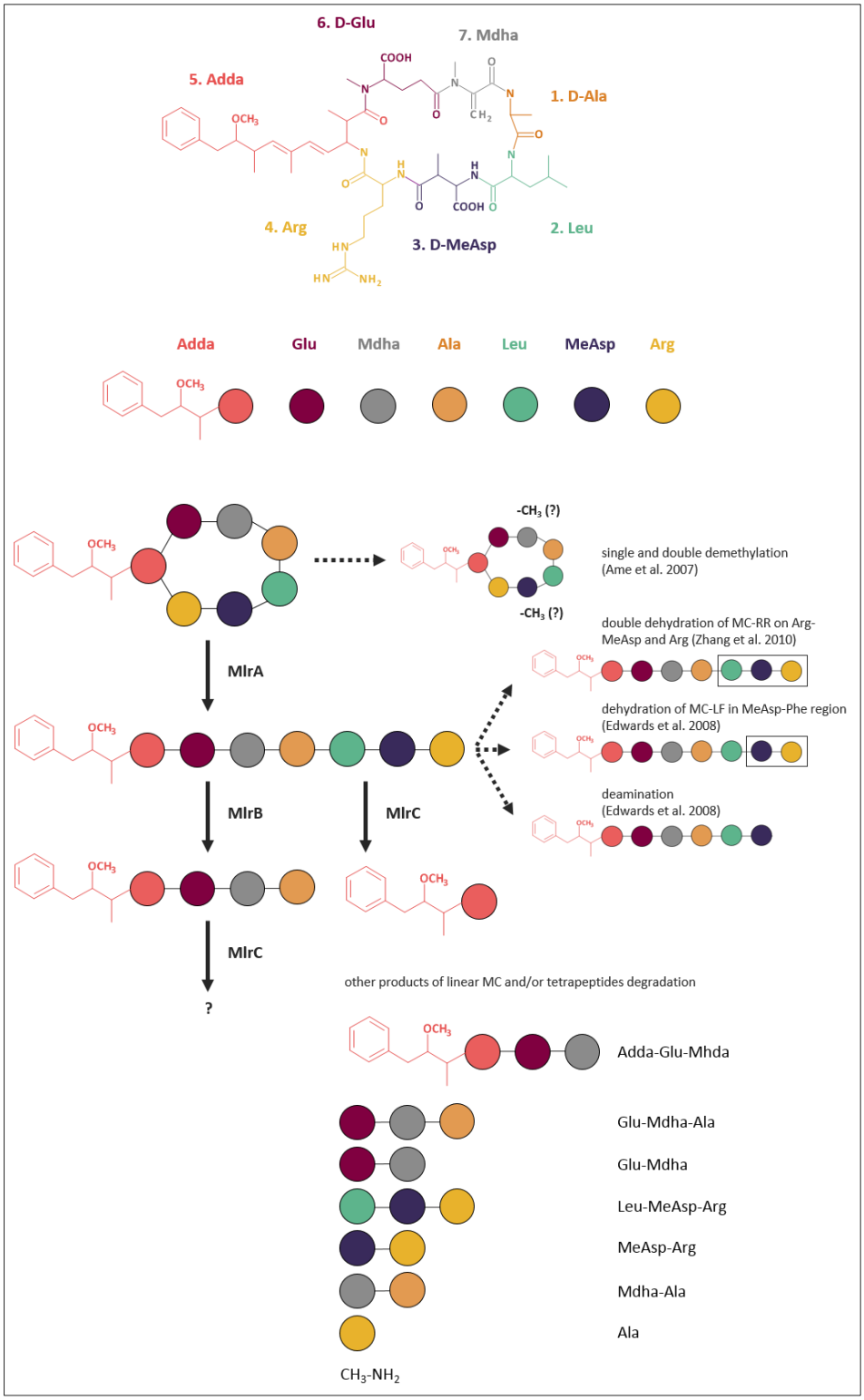

Figure 9. Proposed steps of MC-degradation. Modified after (Dziga et al, 2013)

In **MANUSCRIPT I**, several bacterial species, some of them formerly unrelated to MC-degradation, were found to be enriched in the presence of MC-containing *Microcystis aeruginosa*. These distinct phylogenetic groups were suggested to be potentially associated with MC-degradation, since enrichment of these bacterial groups occurred simultaneously to MC-removal. A complete elucidation of MC-degradation pathways and the enzymes involved may be feasible. Stable isotope probing might be

a reasonable approach to further investigate and describe MC-degrading bacteria, their properties and the involved enzymes (Dziga et al, 2013; Radajewski et al, 2000). For this purpose, cyanobacteria may be cultured in ^{13}C and ^{15}N containing medium, which should result in labelled MCs to allow for identification of MC-degrading bacteria and subsequent tracking and elucidating of MC-degradation pathways in responsible bacteria.

6.2. Serine Protease Inhibitors

Several authors have regarded cyanobacteria as generally toxic, if exposed zooplankton perished more rapidly in their presence than in the absence of food (DeMott et al, 1991). Besides the known MCs, several cyanobacterial serine protease inhibitors were found to be toxic to crustaceans (**MANUSCRIPT II**) (Blom et al, 2003; Gademann et al, 2010; Kaebernick et al, 2001; Portmann et al, 2008; Rohrlack et al, 2004). Yet, besides their common characteristic of inhibiting several serine proteases, the mode of action of these toxins is greatly unknown. Despite the similarities in terms of enzyme inhibiting properties, a wide variety of structural differences among these cyanobacterial peptide classes can be found: rather small compounds with a linear chemical structure such as aeruginosins (**MANUSCRIPT II**) or small compounds with a cyclic structure like cyanopeptolins and aerucyclamides or larger compounds with a more complex cyclic structure such as microviridins (Gademann et al, 2010; Portmann et al, 2008; Rohrlack et al, 2003; Welker & von Döhren, 2006). Thus, similar bioactive properties of particular cyanobacterial compounds are not necessarily reflected by similarities in their chemical structures.

In **MANUSCRIPT IV**, effects of aeruginosin 828A (AG 828A) on *D. magna* included lethal moulting difficulties and softening of the carapace in mainly juvenile animals. These symptoms were similar to effects of microviridin J on *D. pulicaria*. Interestingly, microviridin J was also found in a MC-deficient strain (Kaebernick et al, 2001; Rohrlack et al, 2004). Kaebernick and co-workers (2001) explained these effects of microviridin J with its properties as a serine protease inhibitor. Serine proteases are essential for the metabolization of dihydroxyphenylalanine (DOPA) from tyrosine, which is a crucial precursor of insect cuticular sclerotization: decarboxylation of DOPA to dopamine is catalysed by a serine protease called DOPA-decarboxylase (Hori et al, 1984).

Besides moulting disruption also the down-regulation of vitellogenin (*vtg*) mRNA in juvenile animals was caused by AG 828A (**MANUSCRIPT IV**), which may be interpreted as an endocrine effect (Hannas et al, 2011; Kim et al, 2011). Similar symptoms of deformation together with effects on the *vtg* expression were observed in newborn *Daphnia* when treated with insecticides with (anti-)ecdysteroidal activity (Kim et al, 2011). While susceptibility to AG 828A was observed predominantly in juvenile *Daphnia magna*, the novel toxin might still affect development and moulting in a comparable way as agonists of ecdysteroids and juvenile hormones. However, the mechanisms responsible for the

elevation or suppression of *vtg* in daphnids are not well understood and need further investigation. The production of secondary metabolites with juvenile hormone agonist activity is well known in higher plants and is believed to be part of an insect defence system (Toong et al, 1988).

Nevertheless, the acute toxicity of certain serine protease inhibitors cannot be simply explained by starvation, i.e., via the inhibition of the digestive enzymes in the gut systems. Even more, acute toxicity in *Thamnocephalus platyurus* is routinely tested in early life stages in the absence of food (Palma et al, 2008). Thus, the specific modes of action of serine proteases remain unclear. However, microarray analyses of CP 1020 in zebrafish embryos revealed first hints regarding pathways and cellular targets of CP 1020. These toxins might have some effect on the circadian rhythm and moreover, were suggested to exhibit neurotoxicity (**MANUSCRIPT III**). Thus, CP 1020 in general might affect serine proteases involved in neurological processes, e.g. neurotrypsin or neurotrypsin-like enzymes (Sonderegger, 2013).

7. Possible Functions of Cyanobacterial Peptides

The ability of cyanobacteria to constitutively produce a wide variety of secondary metabolites with a broad bioactivity has puzzled researchers for long time (Welker & von Döhren, 2006). Even more, cyanobacteria from the same genus may produce not only different peptide patterns but may also vary in peptide quantities (Welker et al, 2004a). These so called chemotypes produce a very unique combination of different derivatives of cyanobacterial peptides (Rohrlack et al, 2008). Cyanobacteria seem to invest a large proportion of their energy into the establishment of this characteristic peptide pattern. But what might be the reason for the production of the different peptides, the function of these peptides, and their infinite complexity of combinations?

Given the fact, that cyanobacteria produce a wide variety of phosphatase and protease inhibitors and that these enzymes are crucial for many biological processes, cyanobacteria might have created a potent defence system, which includes competitive benefit (Bajpai et al, 2013). Similar defence mechanisms are known from plants, where secondary metabolites may exhibit diverse functions in reaction to their environment (Jongsma & Bolter, 1997). Despite the many compounds with unknown functions, distinct roles were discovered for some plant derived secondary metabolites, e.g. beneficial compounds for their surrounding potentially mutualistic organisms but mostly repellents, deterrents or antibiotics (Vining, 1990).

The majority of the cyanobacterial peptides is produced via NRPS/PKS pathways that are regulated by genetic and enzymatic parameters and thus, are highly variable. As a result, cyanobacterial peptide composition may change quickly in order to adapt to new biotic and abiotic parameters (Holland & Kinnear, 2013). Therefore, the cyanobacterial peptide composition has possibly developed

over time according to its biological function with MC-producing and MC-deficient strains suggested to represent different ecotypes (Alexova et al, 2011; Tonietto et al, 2012).

7.1. GRAZER DEFENCE

As MCs have been the focus of cyanobacterial research for a long time, different ecological functions have been associated with them. They were suggested to function as metal chelators or infochemicals but mostly they were proposed to be responsible for defence against grazers (Kurmayer & Jüttner, 1999; Rohrlack et al, 1999; Schatz et al, 2007; Utkilen & Gjølme, 1995). However, distinct chemotypes without the ability to produce MCs are well known. They have been suggested to be cheaters in the MC-producer community and thus take advantage of the presence of MC-containing strains in the environment (Ostermaier & Kurmayer, 2009). Interestingly, oxidative stress seems to be one factor promoting MC-producing strains over non-producing strains in natural blooms. Paerl and Otten (2013) reported a selection of toxigenic strains in high-irradiance surface waters and explained their finding with higher resistance of MC-producing strains towards oxidative stressors. During oxidative stress, MCs were found to covalently bind to proteins involved in photosynthesis and carbon sequestration, leading to protein complexes that are resistant to proteolytic degradation (Zilliges et al, 2011). A successional behaviour of increasing concentrations of MC-deficient strains after predominant high densities of MC-producing strains was observed in several lakes during a bloom (Fastner et al, 2001; Welker et al, 2004b; Welker et al, 2007). A lower "critical light intensity" of MC-deficient strains was suggested as a reason for these observations (Kardinaal et al, 2007).

Nevertheless, toxicity against grazers was found also in MC-deficient cyanobacterial strains. Subsequently, peptides compensating the lack of MCs were proposed. Aeruginosins containing chlorine and sulfate are often found in MC-deficient *Planktothrix* strains, and one main representative was characterized in **MANUSCRIPT II**. However, new findings provide evidence that the peptide distribution in *Planktothrix* strains collected from all over the world rather depend on phylogeny, ecophysiological adaptation and geographic distance (Kurmayer et al, 2014). In general, aeruginosins, have been found to not negatively affect mammals, but to rather harm crustaceans (Chlipala et al, 2011). Some crustaceans however, frequently forage on cyanobacteria and were even found to be able to control their abundance in the absence of fish (Sadler & von Elert, 2014).

While it is agreed that MCs exhibit toxicity against grazers, there is genetic proof indicating that MCs have evolved in the ancestors of present-day cyanobacteria long before the first metazoan grazers developed (Rantala et al, 2004). Still, this does not contradict the possibility that the function of MCs and other cyanotoxins have changed towards a defence mechanism against predators due to high grazer pressure (Holland & Kinnear, 2013). However, toxicity of MCs was not only recognized

towards the natural grazers of cyanobacteria but also towards a wide range of non-target organisms including bacteria, protozoans, birds, and mammals (Holland & Kinnear, 2013).

7.2. ALLELOCHEMICALS

Cyanobacterial peptides were suggested to work as allelochemicals, inhibiting growth of competing algae (Leflaive & Ten-Hage, 2007; Yang et al, 2014). The negative effects on co-occurring plants was mostly associated with oxidative stress (Leflaive & Ten-Hage, 2007; Pflugmacher, 2004).

Additionally, MC-producing cyanobacteria were shown to have harmful effects on growth and development of several agricultural important plants by seriously altering their metabolic processes (Abe et al, 1996; Rastogi et al, 2014). Irrigation with cyanobacteria-containing water might therefore pose a problem, especially for the production of economically important plants due to probable reduction of yield quality (Saqrane & Oudra, 2009). Moreover, plants may accumulate toxins and thus increase the risk of poisonings in humans through intake of nutritive plants (Peuthert et al, 2007).

7.3. PARASITE-DEFENCE MECHANISM

Planktothrix communities were proposed to be also shaped by biotic factors such as infections by the omnipresent parasitic chytrid fungi (Rohrlack et al, 2013). These parasites were suggested to lead to successional occurrence of different chemotypes by distinct loss of *Planktothrix* biomass (Rohrlack et al, 2009; Sønstebø & Rohrlack, 2011). Infection of *Planktothrix* by chytrids involves penetration of the cyanobacteria via secretion of digestive enzymes like trypsin and chymotrypsin (Gleason & Lilje, 2009; Symonds et al, 2008). Among the so far discovered cyanobacterial oligopeptides contributing to different chemotypes, many are known as potent serine protease inhibitors, thus impeding penetration of chytrids into cyanobacterial cells. Moreover, intracellular toxins with activity against eukaryotes, may counteract continuation of the chytrid infection (Sønstebø & Rohrlack, 2011).

Wide varieties in chemotype peptide patterns may be explained by Van Valens 'Red Queen' hypothesis (Van Valen, 1973), i.e., evolution is driven strongest by interactions between species, rather than adaptation to the environment, and moreover, narrow host ranges select for diversification in hosts. The resulting population subdivision of a cyanobacterial species into a wide variety of co-existing chemotypes may lead to unequal susceptibility to the parasitic fungi (Sønstebø & Rohrlack, 2011; Wilson & Sherman, 2013).

In addition, the 'killing-the-winner' concept of Thingstad and Lignell (1997) was also considered for explaining the subdivision of cyanobacterial communities into chemotypes (Sønstebø & Rohrlack, 2011). According to this concept, highest infection rates of the most abundant host leads to stable or increased host diversity, thereby forcing host and parasite into co-existence. Thus, Sønstebø and co-workers (2011) suggested parasitic chytrid fungi to be one potential driving force of *Planktothrix* sp. population subdivision and chemotype dynamics.

Besides fungi, viruses like cyanophages are known to infect cyanobacterial communities (Jassim & Limoges, 2013). Viral infection generally requires a wide variety of proteases for penetration, replication and lysis of the host cells (Koltukova et al, 1995). Moreover, resistances of certain cyanobacterial strains towards cyanophages with broad host spectra were observed (Tucker & Pollard, 2005; Watkins et al, 2014). None of these studies took into account the different peptide pattern produced by various cyanobacterial chemotypes. However, it might be reasonable that mechanisms similar to those proposed for interactions of cyanobacteria with chytrids were also acting against viruses. Thus, not only parasitic fungi like chytrids but also viruses like cyanophages may be considered as potential drivers for cyanobacterial oligopeptides diversification.

8. CONCLUSION

High costs and constant production of a wide variety of cyanobacterial secondary metabolites with a broad range of bioactivities may imply a distinct function of these compounds. Regarding the potent toxicity of certain cyanobacterial peptides, a role in protection against predators was suggested. Thus, the diversity of oligopeptides might work as a sophisticated defence system that evolved for approximately 3.5 billion years as safety appliance against co-existing and co-evolving biotic threats such as grazers, fungi, viruses or competing cyanobacteria and algae. Still, the ecophysiology and the broader role of cyanobacterial peptide production as well as the mechanisms involved in chemotype diversification remain to be elucidated. This information would clearly be useful, not only to better understand ecology, biology and physiology of cyanobacteria, but also in order to manage and treat cyanobacterial blooms accordingly to prevent environmental and human risks. Finally, modes of action of cyanobacterial toxins remain to be elucidated, in order to find more appropriate treatments for outbreaks of cyanobacterial intoxications.

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Toxicity of microcystin-deficient Planktothrix *strains due to chlorine and sulfate containing aeruginosins.*
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PUBLICATIONS

Kohler, E., Blom J. F., Faltermann S., Fent K. and Pernthaler J. Effects of aeruginosin 828A, microcystin-LR and cyanopeptolin CP1020 on life-history parameters and selected gene transcripts in *Daphnia magna*. (in preparation)

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